Studies on a Novel System for Cell-free Protein Synthesis Based on the Hyperthermophilic Archaeon, Thermococcus kodakaraensis(Dissertation_全文)

AUTHOR(S):

Endoh, Takashi

Studies on a Novel System for Cell-free Protein Synthesis Based on the Hyperthermophilic Archaeon, *Thermococcus kodakaraensis*

Takashi ENDOH

2008

PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The study in this thesis has been performed under the supervision of Professor Tadayuki Imanaka in the Laboratory of Biochemical Engineering, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University during 2004-2008.

The author would like to express his sincerest gratitude to Professor Tadayuki Imanaka for his invaluable guidance, worthy suggestions, and ceaseless encouragements throughout the course of this study. The author is deeply grateful to Assistant Professor Tamotsu Kanai and Associate Professor Haruyuki Atomi for fruitful discussions, precious suggestions and heartwarming supports about this work.

It should be emphasized that the study in Chapter 1 of this thesis was made possible through a fruitful collaboration. Grateful acknowledgement is dedicated to Professor Kenichi Yoshikawa and Dr. Yuko T. Sato in the Department of Physics, Graduate School of Science, Kyoto University, and Mr. David V. Liu in Department of Chemical Engineering, Stanford University.

The author cannot forget to express his great thanks to all the colleagues, past and present, in the Laboratory of Biochemical Engineering. Without their continuous supports and encouragements, he would not have been able to complete this study.

Finally, the author would deeply appreciate to his parents, Yasuo Endoh and Hisako Endoh, for their continuous affections, and understanding, and heartwarming encouragements, and to his sisters and brothers, Maya, Hitoshi, Emi, Tadanori, Momoko and Florian for their continuous encouragements.

Takashi Endoh

Laboratory of Biochemical Engineering

Department of Synthetic Chemistry and Biological Chemistry

Graduated School of Engineering

Kyoto University

2008

Kyoto

TABLE OF CONTENTS

GENERAL INTRODUCTION

1. Cell-free protein synthesis.

Cell-free protein synthesis (CFPS) is a method to synthesize proteins *in vitro* by using mRNA (or DNA) used as template and the active translation machinery in the cell lysate which contains ribosomes, translation initiation factors, aminoacyl-tRNA synthetases, tRNAs and so on (Fig. 1) (24, 27). The CFPS system was originally developed in the 1950s by Zamecnik and Hoagland using rat liver microsome fraction (16). Using the system, they discovered two important steps in the process of protein synthesis: the activation of amino acids by ATP and their subsequent linkage to an unidentified cellular RNA fraction, which is now called "tRNA". In 1961, Matthaei and Nirenberg deciphered the genetic code of living cells for the first time by using the CFPS system derived from *Escherichia coli* (24). As the CFPS system is able to synthesize proteins with similar speed and accuracy as living cells (26), this was frequently used as a tool to elucidate the cellular mechanisms of protein synthesis, mainly from the 1960s to 1970s (6, 7, 10). However, the CFPS system at the time displayed only short duration times and the yields of protein production were low (27, 40). For those reasons, the CFPS system was not considered as a method to synthesize heterologous proteins.

On the other hand, the gene recombination technology developed in the 1970s made it possible to produce heterologous proteins in living cells, such as *E. coli*, yeast, and mammalian cultured cells (8, 13, 19). At present, recombinant proteins can be obtained at low costs by large-scale cultivation of these genetically engineered-cells. In addition, mutant- or chimera-type proteins showing much higher (or lower) activities can be produced by the development of gene manipulation technology (3). At present,

protein production in genetically engineered-cells has been applied for the production of medicine, cosmetic, food materials etc., and is now an indispensable technology for everyday life. On the other hand, some proteins, although few in number, are difficult to produce in living cells. Examples of these are proteins that display toxicity to host cells or contain unnatural amino acids. These problems derive from the facts that protein synthesis in living cells is dependent on cell growth, or that unnatural amino acids are difficult to permeate into cells through the lipid bilayer membranes.

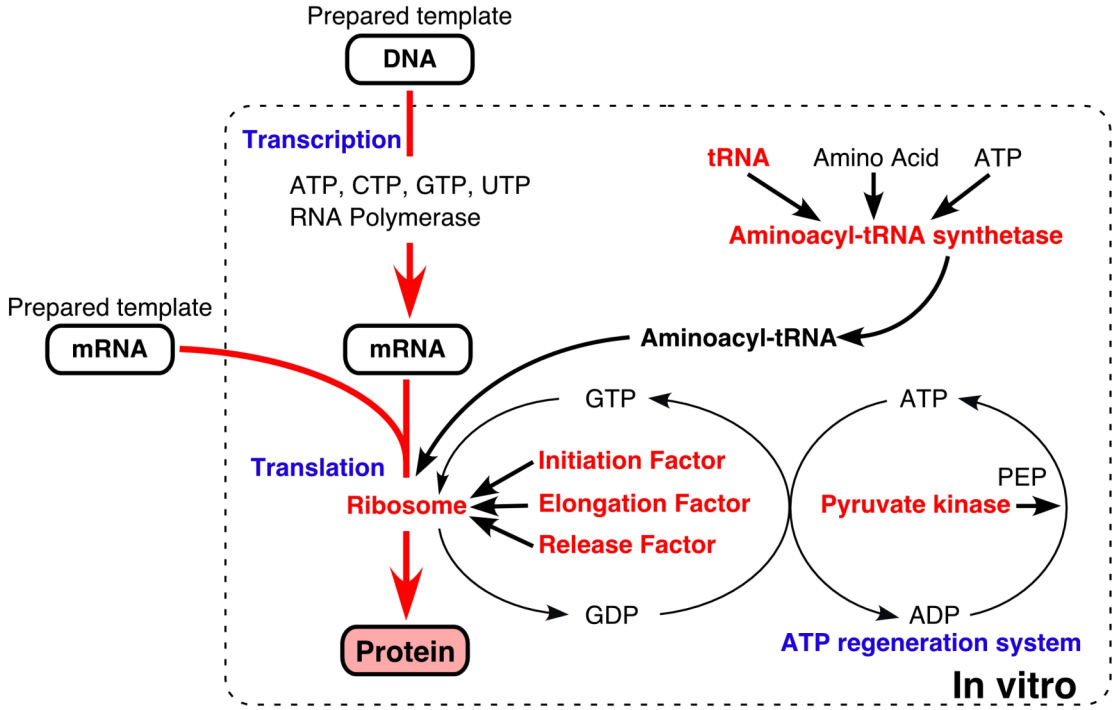

Fig. 1 Schematic drawing of cell-free protein synthesis.
Red characters indicate factors contained in cell-free extract.

As the CFPS system is free from cell constraints, and can be developed and modified exclusively for protein synthesis (Table 1), this system is suitable for the production of the above-mentioned proteins. Moreover, the CFPS system has an advantage in high-throughput protein production because of the following reasons: 1) simple reaction procedure by mixing DNA or mRNA templates with reaction buffer containing cell-free extract, 2) easy purification of products without the need for cell disruption. Based on these standpoints, several researchers continued to work on the CFPS system, aiming to develop the system for use in the field of protein production. In the late 1980s, Spirin and coworkers developed a new reaction system called "continuous flow cell-free (CFCF) translation system" in which a solution containing amino acids and energy sources is supplied continuously to the reaction chamber through a filtration membrane (34). This system made it possible to sustain protein synthesis over 20-40 hours. Together with the continuous-exchange cell-free (CECF) translation system developed afterwards (2), these new systems significantly increased the yields of protein synthesis to a level of mg/mL order. At present, the CFPS system is considered a practical method for protein synthesis, complementing the protein production systems in living cells. However, there still exist some problems, for example, high production cost compared to the system of living cells, and many researches are now in progress to further develop the system (15, 25, 31, 37). There are three types of cell lysates frequently utilized for cell-free protein synthesis, which are as follows: *E. coli* (27), rabbit reticulocyte (14) and wheat germ (11) (Table 2). The *E. coli* system, which has been developed by many research groups, generally gives high productivity among the CFPS systems (21). It is also noteworthy that the completely reconstituted CFPS system (PURESYSTEM) was developed using the *E. coli* system (32). The wheat germ system provides an attractive alternative as a stable eukaryotic

system that can produce relatively a large amount of proteins (22). Although the rabbit reticulocyte system has not been optimized for use in protein production system, this system provides a better platform for functional studies on post-translational modification of eukaryotic proteins (41).

Table 1. Comparisons of proteins production between living cells and the CFPS system

	Productivity	Toxic protein	Modification of system	High throughput	Cost
Living cells	+	−	−	−	+
CFPS system	±	+	+	+	−

+: good ±: fair −: not good

Table 2. Features of *E. coli*, wheat germ and rabbit reticulocyte-based CFPS systems

	E. coli	Rabbit reticulocyte	Wheat germ
Productivity	+	−	±
Post-translational modification	−	+	+
Cost	+	−	+
Optimum reaction temperature	**37 °C**	**30 °C**	**25-30 °C**

+: good ±: fair −: not good

2. Archaea and hyperthermophiles.

All life on the earth is divided into three domains: *Eucarya*, *Bacteria* and *Archaea* (Fig. 2) (39). The domain *Archaea*, proposed by Carl Woese in 1977 as *Archaebacteria* (38), is further classified into two major kingdoms: *Crenarchaeota* and *Euryarchaeota*. Recently, two new kingdoms of this domain have been proposed: *Korarchaeota* (5) and *Nanoarchaeota* (17). Members that constitute the *Archaea* includes many extremophiles, that are organisms living in the extreme environmental conditions, such as (hyper)thermophiles, acidophiles, halophiles, and methanogens.

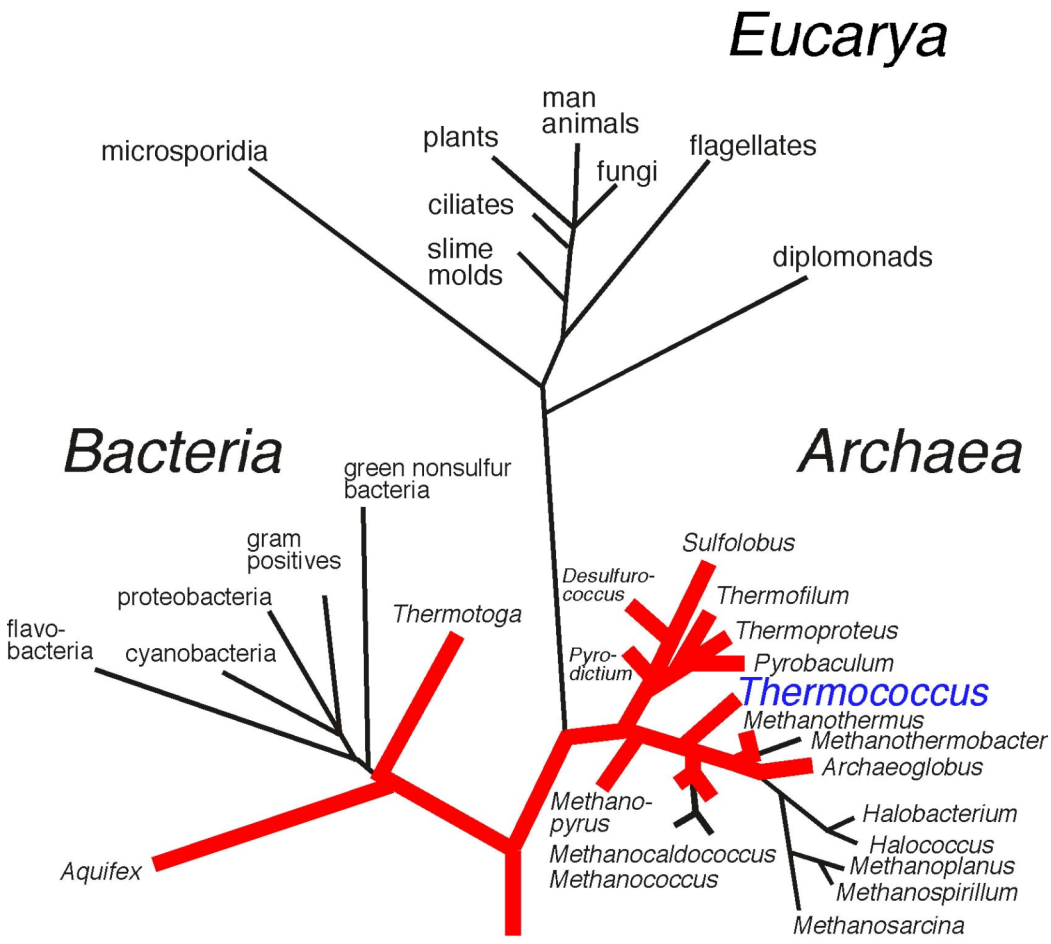

Fig. 2 The phylogenetic tree of life. The red bold line denotes hyperthermophiles.

Hyperthermophiles are generally defined as organisms that grow optimally at temperatures above 80 °C (35). Virtually all hyperthermophiles are members of the domain *Archaea*, while some exceptional members belong to the bacterial orders, *Aquificales* and *Thermotogales*. In the phylogenetic tree of life based on the 16S ribosomal DNA sequences, hyperthermophiles occupy the deepest and shortest branches (Fig. 2), suggesting that they may represent the most primitive forms of present day life. The environments they inhabit (high temperatures, anaerobic atmosphere) also resemble the conditions of our planet in the times when life originated.

Genome analyses of over twenty species of hyperthermophiles showed that their genome size, which ranges from 1.5 to 3.0 Mbp (containing 1,520 to 2,977 ORFs), is relatively small among the members of free-living microorganisms. This fact suggests that hyperthermophiles are one of the simplest forms of life, being an ideal research target to study the basic principles of the biological mechanisms that sustain life.

Studies on hyperthermophiles are also interesting in the aspect of protein application (1). Enzymes and proteins from hyperthermophiles are extremely stable at high temperature and in the presence of protein denaturants (18, 33). Therefore, hyperthermophiles are regarded as a potential source of stable biocatalysts. Moreover, from experimental aspects, proteins of hyperthermophiles are relatively easy to purify and crystallize. One of the most successful applications of these proteins is thermostable DNA polymerases used for PCR (36), which has brought about tremendous progress in the field of gene manipulation.

3. The hyperthermophilic archaeon, *Thermococcus kodakaraensis.*

Thermococcus kodakaraensis KOD1 is a sulfur-reducing hyperthermophilic archaeon isolated from a solfatara on the shore of Kodakara Island, Kagoshima, Japan (4, 23) (Fig. 3). The organism can grow in a wide temperature range between 60 and 100 °C, with an optimal temperature of 85 °C (Table 3). This archaeon belongs to the order Thermococcales in *Euryarchaeota*. Like the other members of this order, *T. kodakaraensis* is an anaerobic heterotroph that utilizes peptide-related substrates with elemental sulfur (S^0) evolving hydrogen sulfide as a reduction endproduct. *T. kodakaraensis* also grows on maltodextrin or pyruvate without S^0, evolving hydrogen gas (20). Cultivation of *T. kodakaraensis* is relatively easy among hyperthermophiles, as this organism grows in a simple medium (such as the one containing artificial sea salt, yeast extract, tryptone, and maltodextrin), to high cell densities (several gram wet cells can be obtained per liter culture), in a short cultivation period (doubling time is less than 1 h at 85 °C).

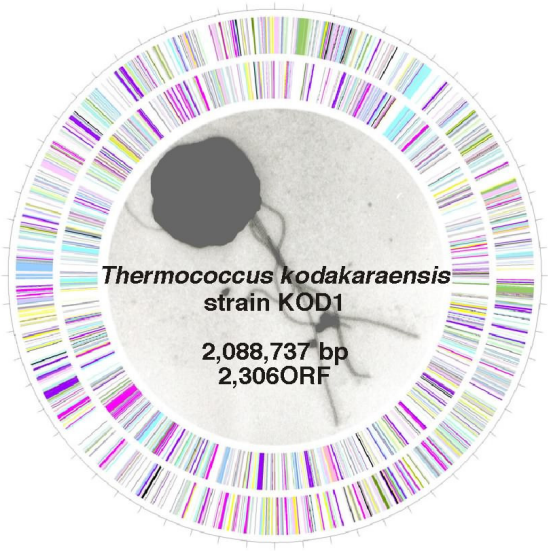

Fig. 3 ***T. kodakaraensis*** **KOD1**

Table 3. Basic properties of *T. kodakaraensis* KOD1

Cell shape	Irregular coccus, Flagellate
Cell size	1μm
Growth condition	Strict anaerobe, Heterotroph
Growth temperature (optimum)	60 – 100 °C (85 °C)
Growth pH (optimum)	5 – 9 (6.5)
Optimum NaCl concentration	3 %
Gram stain	Negative

The complete genome sequence of *T. kodakaraensis* has recently been determined and annotated (12). The genome consists of 2,088,737 bp and is predicted to harbor 2,306 open reading frames (ORFs). Among those, 1,165 ORFs are annotatable, whereas the function of 1,141 ORFs cannot be predicted from the primary structures. Furthermore, *T. kodakaraensis* is one of the few hyperthermophiles that have a practical genetic transformation system (29, 30). These features enable us to undertake molecular breeding of *T. kodakaraensis* through the disruption and/or replacement of any specific genes on the genome.

4. Objectives of this study.

The CFPS systems available today are all based on organisms living at moderate temperatures. Accordingly, protein synthesis reactions of the present systems are performed in a moderate temperature ranging from 20 to 37 °C (Table 2). One of the main objectives of this study is to challenge the upper temperature limit of the CFPS system by using the cell lysate of *T. kodakaraensis*. As *T. kodakaraensis* is a hyperthermophile growing in a temperature range between 60 and 100°C, it is expected that the CFPS system based on this organism works effectively under high temperature

conditions. As biomolecules of hyperthermophiles show high stability, it is also expected that the CFPS system made by *T. kodakaraensis* is robust and thus the duration of the CFPS reaction might be extended compared to conventional systems.

Another objective of this study is to evaluate the protein production potential of the *T. kodakaraensis* CFPS system. There is a study showing that [^{35}S] methionine was incorporated into the protein synthesized *in vitro* using a cell lysate of the hyperthermophilic acidophile, *Sulfolobus solfataricus* strain MT4 (9, 28). Unfortunately, in this case, yields of the protein synthesized were not determined, and the protein production potential of the CFPS system based on hyperthermophiles is still unclear. In this study, the author demonstrates that yields of the protein synthesized by the *T. kodakaraensis* CFPS system can exceed 100 μg/mL. The author also shows successful production of a GFP derivative from a heterologous origin and multiple gene products from a single polycistronic mRNA.

REFERENCES

1. **Adams, M. W. W., and R. M. Kelly.** 1998. Finding and using hyperthermophilic enzymes. *Trends Biotechnol.* **16:**329-332.

2. **Alakhov, Y. B., V. I. Baranov, S. Y. Ovodov, L. A. Ryabova, A. S. Spirin, and I. Y. Morozov.** 1995. Method of preparing polypeptides in a cell-free translation system. United States Patent 5,478,730.

3. **Arai, R., W. Wriggers, Y. Nishikawa, T. Nagamune, and T. Fujisawa.** 2004. Conformations of variably linked chimeric proteins evaluated by synchrotron X-ray small-angle scattering. *Proteins* **57:**829-838.

4. **Atomi, H., T. Fukui, T. Kanai, M. Morikawa, and T. Imanaka.** 2004. Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic

archaeon previously reported as *Pyrococcus* sp. KOD1. *Archaea* **1:**263-267.

5. **Barns, S. M., C. F. Delwiche, J. D. Palmer, and N. R. Pace.** 1996. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proc. Natl. Acad. Sci. USA* **93:**9188-9193.

6. **Cenatiempo, Y., T. Twardowski, B. Redfield, B. R. Reid, H. Dauerman, H. Weissbach, and N. Brot.** 1983. Simplified *in vitro* system for study of eukaryotic mRNA translation by measuring di- and tripeptide formation. *Proc. Natl. Acad. Sci. USA* **80:**3223-3226.

7. **Clark, V. L.** 1980. Inhibition of the initiation of translation by a factor isolated from *Escherichia coli* cells. *Proc. Natl. Acad. Sci. USA* **77:**1181-1184.

8. **Cohen, S. N., A. C. Chang, H. W. Boyer, and R. B. Helling.** 1973. Construction of biologically functional bacterial plasmids *in vitro*. *Proc. Natl. Acad. Sci. USA* **70:**3240-3244.

9. **Condo, I., A. Ciammaruconi, D. Benelli, D. Ruggero, and P. Londei.** 1999. *Cis*-acting signals controlling translational initiation in the thermophilic archaeon *Sulfolobus solfataricus*. *Mol. Microbiol.* **34:**377-384.

10. **Das, A., I. P. Crawford, and C. Yanofsky.** 1982. Regulation of tryptophan operon expression by attenuation in cell-free extracts of *Escherichia coli*. *J. Biol. Chem.* **257:**8795-8798.

11. **Endo, Y., and T. Sawasaki.** 2006. Cell-free expression systems for eukaryotic protein production. *Curr. Opin. Biotechnol.* **17:**373-380.

12. **Fukui, T., H. Atomi, T. Kanai, R. Matsumi, S. Fujiwara, and T. Imanaka.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. *Genome Res.* **15:**352-363.

13. **Goeddel, D. V., D. G. Kleid, F. Bolivar, H. L. Heyneker, D. G. Yansura, R. Crea, T. Hirose, A. Kraszewski, K. Itakura, and A. D. Riggs.** 1979. Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proc. Natl. Acad. Sci. USA* **76:**106-110.

14. **Hempel, R., J. Schmidt-Brauns, M. Gebinoga, F. Wirsching, and A. Schwienhorst.** 2001. Cation radius effects on cell-free translation in rabbit reticulocyte lysate. *Biochem. Biophys. Res. Commun.* **283:**267-272.

15. **Henrich, B., W. Lubitz, and R. Plapp.** 1982. Lysis of *Escherichia coli* by induction of cloned ϕ X174 genes. *Mol. Gen. Genet.* **185:**493-497.

16. **Hoagland, M. B., P. C. Zamecnik, and M. L. Stephenson.** 1957. Intermediate reactions in protein biosynthesis. *Biochim. Biophys. Acta.* **24:**215-216.

17. **Huber, H., M. J. Hohn, R. Rachel, T. Fuchs, V. C. Wimmer, and K. O. Stetter.** 2002. A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* **417:**63-67.

18. **Imanaka, T., and H. Atomi.** 2002. Catalyzing "hot" reactions: enzymes from hyperthermophilic Archaea. *Chem. Rec.* **2:**149-163.

19. **Itakura, K., T. Hirose, R. Crea, A. D. Riggs, H. L. Heyneker, F. Bolivar, and H. W. Boyer.** 1977. Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* **198:**1056-1063.

20. **Kanai, T., H. Imanaka, A. Nakajima, K. Uwamori, Y. Omori, T. Fukui, H. Atomi, and T. Imanaka.** 2005. Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. *J. Biotechnol.* **116:**271-282.

21. **Kim, T. W., D. M. Kim, and C. Y. Choi.** 2006. Rapid production of milligram quantities of proteins in a batch cell-free protein synthesis system. *J.*

Biotechnol. **124:**373-380.

22. **Madin, K., T. Sawasaki, T. Ogasawara, and Y. Endo.** 2000. A highly efficient and robust cell-free protein synthesis system prepared from wheat embryos: plants apparently contain a suicide system directed at ribosomes. *Proc. Natl. Acad. Sci. USA* **97:**559-564.

23. **Morikawa, M., Y. Izawa, N. Rashid, T. Hoaki, and T. Imanaka.** 1994. Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. *Appl. Environ. Microbiol.* **60:**4559-4566.

24. **Nirenberg, M. W., and J. H. Matthaei.** 1961. The dependence of cell-free protein synthesis in *E. coli* upon naturally occurring or synthetic polyribonucleotides. *Proc. Natl. Acad. Sci. USA* **47:**1588-1602.

25. **Noren, C. J., S. J. Anthony-Cahill, M. C. Griffith, and P. G. Schultz.** 1989. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244:**182-188.

26. **Pavlov, M. Y., and M. Ehrenberg.** 1996. Rate of translation of natural mRNAs in an optimized *in vitro* system. *Arch. Biochem. Biophys.* **328:**9-16.

27. **Pratt, J. M.** 1984. Coupled transcription-translation in procaryotic cell-free systems, p. 179-207. *In* B. D. Hames and S. J. Higgins (ed.), Transcription and Translation; A Practical Approach. IRL Press, Oxford.

28. **Ruggero, D., R. Creti, and P. Londei.** 1993. In vitro translation of archaeal natural mRNAs at high temperature. *FEMS Microbiol. Lett.* **107:**89-94.

29. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2005. Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. *Appl. Environ. Microbiol.* **71:**3889-3899.

30. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2003. Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J. Bacteriol.* **185:**210-220.

31. **Shimizu, M., T. Ikegami, K. Akiyama, and E. H. Morita.** 2006. A novel way to express proline-selectively labeled proteins with a wheat germ cell-free protein synthesis system. *J. Biochem. (Tokyo)* **140:**453-456.

32. **Shimizu, Y., A. Inoue, Y. Tomari, T. Suzuki, T. Yokogawa, K. Nishikawa, and T. Ueda.** 2001. Cell-free translation reconstituted with purified components. *Nat Biotechnol* **19:**751-755.

33. **Shiraki, K., S. Nishikori, S. Fujiwara, H. Hashimoto, Y. Kai, M. Takagi, and T. Imanaka.** 2001. Comparative analyses of the conformational stability of a hyperthermophilic protein and its mesophilic counterpart. *Eur. J. Biochem.* **268:**4144-4150.

34. **Spirin, A. S., V. I. Baranov, L. A. Ryabova, S. Y. Ovodov, and Y. B. Alakhov.** 1988. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **242:**1162-1164.

35. **Stetter, K. O.** 1996. Hyperthermophilic procaryotes. *FEMS Microbiology Reviews* **18:**149-158.

36. **Takagi, M., M. Nishioka, H. Kakihara, M. Kitabayashi, H. Inoue, B. Kawakami, M. Oka, and T. Imanaka.** 1997. Characterization of DNA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR. *Appl. Environ. Microbiol.* **63:**4504-4510.

37. **Vinarov, D. A., B. L. Lytle, F. C. Peterson, E. M. Tyler, B. F. Volkman, and J. L. Markley.** 2004. Cell-free protein production and labeling protocol for NMR-based structural proteomics. *Nat. Methods* **1:**149-153.

38. **Woese, C. R., and G. E. Fox.** 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl. Acad. Sci. USA* **74:**5088-5090.

39. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. USA* **87:**4576-4579.

40. **Yang, H. L., L. Ivashkiv, H. Z. Chen, G. Zubay, and M. Cashel.** 1980. Cell-free coupled transcription-translation system for investigation of linear DNA segments. *Proc. Natl. Acad. Sci. USA* **77:**7029-7033.

41. **Zhang, J. T., M. Chen, C. I. Foote, and B. J. Nicholson.** 1996. Membrane integration of in vitro-translated gap junctional proteins: co- and post-translational mechanisms. *Mol Biol Cell* **7:**471-482.

SYNOPSIS

Recently, cell-free protein synthesis has been the focus of considerable attention, because the cell-free systems offer several advantages over traditional cell-based expression methods, including the easy modification of reaction conditions, decreased sensitivity to product toxicity and suitability for high-throughput strategies. Conventional cell-free protein synthesis systems are based on *E. coli*, wheat embryo or rabbit reticulocyte, and reactions for cell-free protein synthesis are performed at moderate temperatures (20-37 °C). In this study, the author aims to construct a cell-free protein synthesis system that can be operated at high temperatures using cell-free extract of the hyperthermophilic archaeon, *Thermococcus kodakaraensis*. PART I describes the construction and improvement of the cell-free protein synthesis system based on *T. kodakaraensis*. PART II deals with its application to the field of heterologous protein production, and to the synthesis of multiple protein products from a single polycistronic mRNA.

PART I

In Chapter 1, the author constructed a novel system for cell-free protein synthesis that can be operated at high temperatures using a lysate of the hyperthermophilic archaeon, *T. kodakaraensis*. With the system, cell-free protein synthesis of ChiAΔ4, a derivative of *T. kodakaraensis* chitinase, was observed within a temperature range of 40 °C to 80 °C, with an optimum at 65 °C. Corresponding chitinase activity was also detected in the reaction mixtures after cell-free protein synthesis, indicating that the synthesized ChiAΔ4 folded in a proper tertiary structure. The maximum concentration of active ChiAΔ4 synthesized was determined to be

approximately 1.3 μg/mL. A time course experiment indicated that the amount of synthesized ChiAΔ4 saturated within 30 min at 65 °C, and energy depletion was suggested to be the main cause of this saturation. The author further developed the system for a transcription and translation-coupled system at high temperatures using a combination of *T. kodakaraensis* lysate and a thermostable T7 RNA polymerase.

In Chapter 2, the author modified the cell-free protein synthesis system based on *T. kodakaraensis* to increase its productivity through the following approaches. First, the process of lysate preparation was examined, and it was found that omitting the pre-incubation (runoff) step is especially effective to increase the translational activity of lysate. Second, concentrations of each reaction mixture were optimized. Among them, the requirement of a high concentration of potassium acetate (250 mM) was characteristic to the *T. kodakaraensis* system. Third, a mutant strain of *T. kodakaraensis* was constructed in which a heat shock transcriptional regulator gene, *phr*, was disrupted. By using the lysate made from the mutant, an increase in the optimum reaction temperature was observed by 5 °C. Through these modifications to the system, the yield of ChiAΔ4 was dramatically increased to 115.4 μg/mL in a batch reaction at 65 °C, which was about 90 times higher than before. Moreover, in the optimized system, a high speed of protein synthesis was achieved: over 100 μg/mL of ChiAΔ4 was produced in the first 15 min of reaction. These results indicate that the system for cell-free protein synthesis based on *T. kodakaraensis* lysate has a high production potential comparable to the *E. coli*-based system.

PART II

In Chapter 3, the author examined the performance of cell-free protein synthesis system based on *T. kodakaraensis* lysate in the synthesis of green fluorescent

protein (GFP), in order to apply this system to practical use in the field of heterologous protein production. As the wild-type GFP is a thermolabile protein, a thermostable GFP derivative (tGFP) was selected as a candidate for protein synthesis. The first attempt of tGFP synthesis at 60 °C using the system resulted in a detection of small amount of protein (<0.1 μg/mL) by Western blot analysis. Using a newly synthesized tGFP gene in which codon usage was optimized for *T. kodakaraensis* as a template, the synthesis of tGFP was clearly detectable at temperatures between 50 and 65 °C. The tGFP production was further enhanced over 10 μg/mL with the addition of stem-loop structure at the 3'-end of mRNA. Determination of fluorescences of tGFP in the reaction mixtures indicated that active tGFP constituted *ca*. 30 % of the total protein synthesized. Addition of *T. kodakaraensis* chaperonin to the system significantly increased the ratio of active tGFP content to *ca*. 50 %. Through these approaches to the system, the production of tGFP increased over 100-fold, and the yield of active tGFP synthesized reached to 6.5 μg/mL.

In Chapter 4, the author tested whether multiple gene products could be synthesized from a single polycistronic mRNA using the *T. kodakaraensis* cell-free translation system. Acetyl-CoA synthetase III of *T. kodakaraensis* (ACSIII), which is a heterotetmeric enzyme composed of two α- and two β-subunits, is encoded by a single gene operon (TK0944-TK0943), and used as the target protein. For detection and purification of the proteins synthesized, template genes were modified to attach a His-tag to the amino-terminus of TK0944 protein (His-TK0944), and a HA-tag to the carboxyl-terminus of TK0943 protein (TK0943-HA). When the reaction products were purified using columns that selectively attach the His-tag proteins, His-TK0944 protein was found in the purified fraction. On the other hand, TK0943-HA protein did not co-purified with the His-TK0944 protein by the purification, although synthesis of the

TK0943-HA protein in the reaction mixture was confirmed by Western blot analysis. These results indicated that both gene products (His-TK0944 and TK0943-HA) were successfully synthesized from a single polycistronic mRNA by the *T. kodakaraensis* cell-free translation system.

PART I

Development of the cell-free protein synthesis system Using a lysate of *Thermococcus kodakaraensis*.

CHAPTER 1

Cell-free protein synthesis at high temperatures using the lysate of a hyperthermophile

INTRODUCTION

The production of recombinant proteins in appropriate host cells is now a routine alternative for studying the function and biophysical properties of a given protein. The variety of host cells available has expanded greatly in recent years, and ranges from the bacterial and archaeal prokaryotic cells to the higher eukaryotic cells. However, recombinant protein production in living-cells sometimes shares a common drawback when the target protein is toxic and/or incompatible with host cell growth. This often leads to growth retardation of the host strain, low protein yield, or destabilization of the expression vector (2, 8, 16).

Cell-free protein synthesis is a method to synthesize proteins *in vitro* by using mRNA and the active translation machinery in the cell lysate (4, 17). One of the advantages of this system is that one can utilize and develop the system focusing only on protein synthesis *per se*, and therefore, highly toxic proteins can readily be produced with *in vitro* systems (10). Another major advantage is that these systems, with the properly charged tRNAs, allow the synthesis of proteins containing unnatural amino acids (19). Other notable features are the relatively short periods of time required for protein synthesis and the rather simple purification procedure following protein synthesis.

At present, there are three major sources of lysates utilized for cell-free protein synthesis: *Escherichia coli* (28), rabbit reticulocyte (9) and wheat germ (6). As

these lysates originate from organisms living at moderate temperatures, protein synthesis reactions are performed in a temperature range between 20 °C and 40 °C. Although these systems can be presumed to be sufficient for producing a majority of mesophilic proteins, there are several reasons for one to explore the possibilities of protein synthesis at higher temperatures. A slight elevation in temperature, to an extent that it does not denature the target protein itself, will lead to more rapid protein synthesis. It has been reported that, by using capped mRNA, the reaction temperature of wheat germ extract could be increased up to 37 °C (from 20 °C), and an increased amount of protein synthesis was observed as a result of high speed protein synthesis (31). In addition, elevated temperatures can be expected to prevent mRNA secondary structures that otherwise might be inhibitory in the translation reaction (18).

In order to develop an *in vitro* translation system that functions and exhibits stability at elevated temperatures, the use of (hyper)thermophiles as a source of cell lysate is a practical choice. The *in vitro* incorporation of [^{35}S] methionine into proteins has previously been reported using the lysate of *Sulfolobus solfataricus* strain MT4 (3, 23), suggesting that the lysates from hyperthermophiles have the potential to be utilized for *in vitro* translation systems. Besides the stability at moderately high temperatures (~50 °C), development of this type of system using the lysate of a hyperthermophile would greatly expand the temperature range at which cell-free protein synthesis can be performed. This should also make possible the production of highly thermostable proteins that cannot be properly folded at ambient temperature.

The author reports here the development of a system for cell-free protein synthesis using a lysate of *Thermococcus kodakaraensis*. *T. kodakaraensis* can grow between 60 and 100 °C with an optimal growth temperature of 85 °C. The broad temperature range at which this organism grows can be expected to provide an

advantage in developing a cell-free system that can function at various extents of elevated temperature. In this chapter, the author has performed an initial examination of various parameters and components that affect the rate and yield of protein synthesis, and with this system the author has been able to observe the *in vitro* production of an active protein at temperatures between 40 and 80 °C.

MATERIALS AND METHODS

Chemicals.

Sulfur, Tris-acetate, ammonium acetate, polyethyleneglycol 8000 (PEG8000) and potassium phosphoenolpyruvate (PEP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP, GTP, CTP and UTP were from Sigma (St. Louis, MO, USA). RNase inhibitor RNAsecure™ from Ambion (Austin, Texas, USA). All the other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

Plasmids and mRNA preparation.

The template DNA, pTRC1, used for preparing ChiAΔ4 mRNA, was constructed as follows. The XbaI site of pUC118 was removed with the Blunting High kit (Toyobo, Osaka, Japan) beforehand, and a BglII-EcoRI fragment (150 bp) containing a T7 promoter was excised from pET-21a(+) (Novagen, Darmstadt, Germany) and inserted between BamHI and EcoRI sites of pUC118. The resulting plasmid was named pT1. A 45 bp-DNA fragment containing the ribosome-binding site of the *T. kodakaraensis* glutamate dehydrogenase gene (21) was synthesized by a polymerase reaction using the following two primers: GDH-R (5'-AAAATCTAGACG CAGATTACCGAAATGAGGT-3', underlined sequences correspond to XbaI site) and GDH-F (5'-AAAACATATGTACCACCTCATTTCGGTAATCTGCG-3', underlined

sequences correspond to NdeI site). The DNA fragment was treated with XbaI and NdeI and inserted into the respective sites of pT1, resulting in the plasmid pT2. A 1,283 bp-DNA fragment containing the ChiAΔ4 gene was amplified with genomic DNA of *T. kodakaraensis* KOD1 by PCR using the following two primers, ChiA-Nd (5'-AAAACATATGCTTCCCGAGCACTTCTTCGCCC-3', underlined sequences correspond to NdeI site) and ChiA-T1 (5'-AAAAGAATTCTCCAATTTCATT ATGGAC-3', underlined sequences correspond to EcoRI site). After treatment with NdeI and EcoRI, the amplified fragment was inserted into the respective sites of pT2, to make pTRC1 (Fig. 1). mRNA encoding ChiAΔ4 was prepared with the T7 RiboMAX^{TM} Express RNA system (Promega, Madison, WI, USA) using pTRC1 as a template. The synthesized mRNA was suspended in RNase-free water and stored at -80 °C until use.

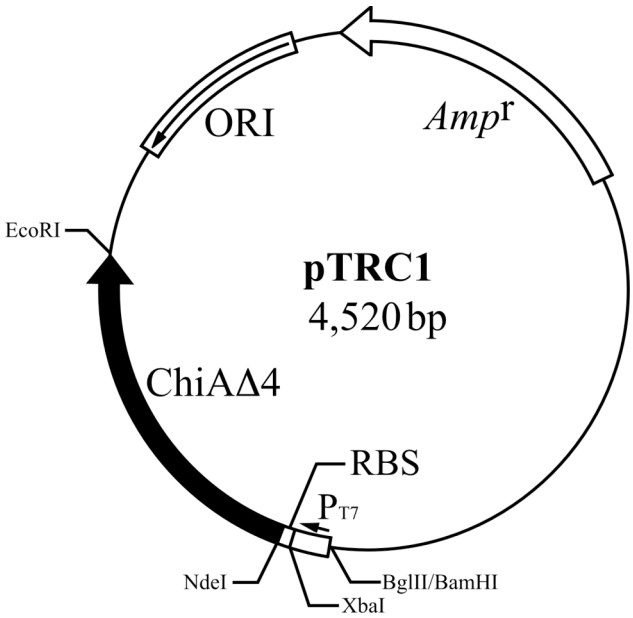

Fig. 1 Schematic drawing of pTRC1 for preparation of ChiAΔ4 mRNA and for the transcription and translation-coupled reaction. pTRC1 was used as a template for these reactions after treatment with EcoRI.

Construction of *T. kodakaraensis* Δ*chiA* strain.

Disruption of *chiA* by double-crossover homologous recombination was performed using the technique developed for *T. kodakaraensis* as described previously (24-26). The vector used for disruption of *chiA* was constructed as follows. A DNA fragment containing the *chiA* coding region together with its flanking regions (about 1,000 bp) was amplified with the primer sets PCHI-R (5'-ACGAACCTTAT TCCTTCTGCATAC-3') and PCHI-F (5'-GGTCAAACTGGAACTGCAACTGCC-3') using genomic DNA of *T. kodakaraensis* KOD1 as a template, and inserted into the HincII site of pUC118. Using the constructed plasmid DNA as a template, the flanking regions of *chiA* along with the plasmid backbone were amplified using primers PDCHIA-R (5'-ACAACACCCCTTGAGCTTTG-3') and PDHIA-F (5'-TTCCCGAGC ACTTCTTCGCCC-3'), and the amplified fragment was designated as L-ChiA. A PvuII-PvuII restriction fragment (763 bp) containing the *pyrF* marker gene was excised from pUD2 (24), and ligation was performed with L-ChiA to construct the plasmid for *chiA* disruption (pUChiA). A *T. kodakaraensis* uracil-auxotroph strain, KU216 (24), was used as a host cell for transformation, and *pyrF*$^{+}$ strain with uracil prototrophy was selected. Whether successful recombination had occurred was checked by PCR, and the constructed strain was named KC1.

Preparation of *T. kodakaraensis* S30 extract.

T. kodakaraensis KC1 was precultured at 85 °C for 12 h in a nutrient-rich medium (MA-YT) (12) containing 0.5% (w/v) elemental sulfur under anaerobic conditions. The preculture was used to inoculate 800 mL culture with MA-YT medium supplemented with 0.5 %(w/v) sodium pyruvate. This was cultured under anaerobic conditions at 85 °C for about 14 h until A_{660} reached 0.6-0.7. Cells were harvested by

centrifugation at 3,000 *g* for 15 min and washed two times with 0.8x Marine Art SF solution (Senju pharmaceuticals, Osaka, Japan) and once with S30 buffer (10 mM Tris-acetate pH 7.4, 1 mM dithiothreitol, 1.4 mM magnesium acetate, and 6.0 mM potassium acetate) supplemented with 0.05% (v/v) 2-mercaptoethanol.

Preparation of S30 extract was performed by a modification of the Pratt method (20) under RNase-free conditions. Cells were suspended in S30 buffer (1.27 mL per gram of wet cells) and disrupted with French Press (FA-003, Thermo Electron Co., Waltham, MA, USA) with a pressure of 10,000 psi. Dithiothreitol was added to the resulting lysate to a final concentration of 1 mM. The lysate was then centrifuged at 30,000 *g* at 4 °C. The upper four-fifths of the supernatant was collected, and a second 30,000 *g* centrifugation was repeated, again collecting only the upper four-fifths of the supernatant. For each 1 mL of the supernatant collected, 0.3 mL of pre-incubation mixture (300 mM Tris-acetate pH 7.4, 9.3 mM magnesium acetate, 13 mM ATP pH 7.0, 84 mM PEP, 0.4 mM dithiothreitol, 1 mM each of 20 amino acids, and 10 units/mL of pyruvate kinase from rabbit muscle (Sigma)) was added, and the mixture was incubated for 80 min at 37 °C. The mixture was then dialyzed three times (45 min each) against 40 times volume of S30 buffer using 5,000 MWCO dialysis tubes. After centrifugation at 4,000 *g* for 10 min, the resulting supernatant was used as the S30 extract. Protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. The S30 extract was stored at -80 °C until use.

Cell-free protein synthesis.

Cell-free protein synthesis was performed in a 30 μL batch reaction containing ChiAΔ4 mRNA, *T. kodakaraensis* S30 extract (8.0 mg/mL, final concentration) and other ingredients shown in the "initial condition" column of Table 1. The reaction was incubated for 90 min at 48 °C and terminated by chilling the reaction on ice. In optimizing the cell-free protein synthesis reaction, the ChiAΔ4 mRNA concentration was first varied (0, 0.2, 0.3 or 0.4 mg/mL). Next, with 0.4 mg/mL mRNA added, the concentration of each component was changed within the ranges shown below: 0, 5, 10, 15, 20 or 25 mM for magnesium acetate; 0, 100, 400 or 700 mM for potassium acetate; 0 or 80mM for ammonium acetate; 0 or 56 mM for Tris-acetate (pH 7.4); 0, 1.2 or 2.4 mM for ATP; 0 or 0.85 mM (each) for GTP, CTP and UTP mixture (GCU mix); 0, 30 or 60 mM for PEP; 0, 2.5, 5.0 %(w/v) for PEG8000; 0, 2 or 4 mM (each) for mixture containing 20 amino acids (20AA mix). With the optimized reaction composition summarized in Table 1, the reaction temperature was examined between 30 °C and 80 °C. The addition of 0.165 mg/mL *T. kodakaraensis* tRNA prepared with the Nucleobond AX kit (Genetics, Düren, Germany) was also tested. The degree of cell-free protein synthesis in the reactions at 60 °C, 65 °C, 70 °C and 80 °C were examined for various time periods between 0 and 120 min. Also, the effect of adding each of the following reagents to the reaction after 45 min at 65 °C was tested: 12 μg mRNA, 36 nmol ATP, and 2.0 μmol PEP.

Transcription-translation coupled reactions were performed with a 30 μL reaction volume containing 2.0 μg of pTRC1, 0-1,500 units of Thermo T7 RNA polymerase (Toyobo), *T. kodakaraensis* S30 extract (8.0 mg/mL, final concentration) as well as the following ingredients: 56 mM Tris-acetate (pH 7.4), 7.5 mM magnesium acetate, 80 mM ammonium acetate, 100 mM potassium acetate, 1.2 mM ATP, 0.85 mM

each of GTP, CTP, and UTP, 30 mM PEP, 2.0 mM each of 20 amino acids, 5 %(w/v) PEG8000. The reaction was performed at 40 °C for the first 60 min, and continued at 60 °C for another 90 min.

Table 1. Composition of reaction mixture for cell-free translation

Component	Unit	Initial condition [*1]	Optimized condition [*2]	Necessity
$Mg(OAc)_2$ [*3]	mM	16	5-10 (0–25)	Yes
K(OAc) [*3]	mM	230	100 (0–700)	No
$NH_4(OAc)$ [*3]	mM	80	80 (0, 80)	No
Tris-acetate (pH 7.4)	mM	56	56 (0, 56)	No
ATP	mM	1.2	1.2 (0–2.4)	No [*8]
GCU mix [*4]	mM (each)	0.85	0.85 (0, 0.85)	No [*8]
PEP [*5]	mM	30	30 (0–60)	Yes
PEG8000 [*6]	%(w/v)	2.0	5.0 (0–5.0)	No
20AA mix [*7]	mM (each)	2.0	2.0 (0-4.0)	Yes
mRNA	mg/ml	0–0.4	0.4 (0–0.4)	Yes
S30 extract	mg/ml	8	8	Yes

[*1] Result is shown in Fig. 2.

[*2] The range over which reactant concentrations were optimized is shown in parenthesis and more precisely in Materials and Methods.

[*3] OAc = acetate

[*4] GTP, CTP and UTP mixture

[*5] Phosphoenolpyruvate

[*6] Polyethyleneglycol 8000

[*7] Mixture containing 20 amino acids

[*8] The presence of one of these components is necessary

Western blot analysis.

After incubation, the reaction mixture was subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5 % acrylamide concentration) followed by blotting to a polyvinylidene fluoride membrane (Hybond^{TM}-P, Amersham Biosciences, Buckinghamshire, UK). Rabbit anti-ChiAΔ4 antiserum was used as the first antibody (1:100,000 dilution), and HRP-rec-Protein G (Zymed Laboratories, San Francisco, CA, USA) was used as the second antibody (1:100,000 dilution). For detection, the ECL Advance^{TM} Western Blotting Detection System (Amersham Biosciences), Hyperfilm^{TM} (Amersham Biosciences) and Lumi vision PRO 400EX (AISIN, Aichi, Japan) were used.

Enzyme assay.

A chitinase activity assay was performed according to the procedure described previously (29) using a fluorometric substrate, 4-methylumbelliferyl β–D-*N*,*N*'-diacetyl chitobioside (Sigma). After a 30 min reaction at 90 °C, the fluorescence of liberated 4-methylumbelliferone was measured (excitation: ca. 365 nm, emission: 460 nm) with a NanoDrop ND-3300 Fluorospectrometer (NanoDrop Technologies, Wilmington, NC, USA). Amount of active ChiAΔ4 synthesized was calculated using the specific activity of purified ChiAΔ4 (0.135 nmol min^{-1} μg^{-1}).

RESULTS

Selection of the target protein.

ChiAΔ4, a truncated form of *T. kodakaraensis* chitinase (29), was selected as the target protein to be synthesized in the *T. kodakaraensis* cell-free translation system. Chitinase from *T. kodakaraensis* (ChiA) contains two catalytic domains (29, 30).

ChiAΔ4 (33.8 kDa) is a ChiA derivative containing only the C-terminal endochitinase domain. As ChiAΔ4 is a highly thermostable enzyme with a half life of over 3 h at 100 °C (29), the effect of heat on protein denaturation during cell-free protein synthesis is negligible. Moreover, as ChiAΔ4 is a protein originating from *T. kodakaraensis*, there is no need for concern with codon preference during protein synthesis. In order to remove any possible effects of the native chitinase present in the cell-free extract, a *chiA*-disrupted strain of *T. kodakaraensis* was constructed and used for preparation of the cell lysate (S30 extract).

Cell-free protein synthesis at high temperatures.

In the preparation of the S30 extract of *T. kodakaraensis*, the auther employed the protocol of Pratt for the preparation of *E. coli* lysate (20), with some modifications. A comparison of the amounts of protein synthesized with different concentrations of ChiAΔ4-encoding mRNA is shown in Figure 2. Western blot analysis revealed that ChiAΔ4 was synthesized only in reactions where exogenous mRNA was added, and that there was a clear correlation between the amount of mRNA added and that of ChiAΔ4 synthesized (Fig. 2).

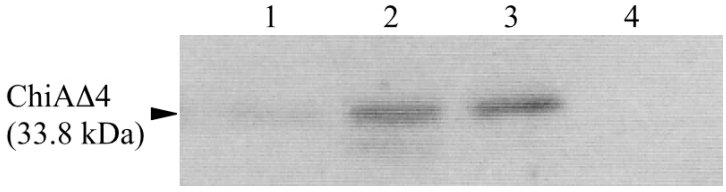

Fig. 2 Cell-free protein synthesis using *T. kodakaraensis* S30 extract. Reaction mixtures containing 0.2 mg/mL of ChiAΔ4 mRNA (lane 1); 0.3 mg/mL of ChiAΔ4 mRNA (lane 2); 0.4 mg/mL of ChiAΔ4 mRNA (lane 3); in the absence of mRNA (lane 4, negative control) were used. Compositions of the reaction mixtures are indicated in Table 1. Reactions were performed at 48 °C for 90 min and ChiAΔ4 was visualized by rabbit anti-ChiAΔ4 antibodies.

Optimization of the reaction mixture composition.

It is well known that protein synthesis in cell-free systems is largely affected by the concentration of reaction constituents (31). The author therefore examined the optimum concentration of each reaction component within the ranges shown in Table 1. In addition to mRNA, S30 extract and the 20 amino acids mixture, the author found that magnesium ions and PEP were necessary for protein synthesis. Furthermore, the presence of either ATP or GCU mix was also found to be necessary for ChiAΔ4 synthesis. The optimized mixture composition was determined to be as follows: 5-10 mM magnesium acetate, 100 mM potassium acetate, 80 mM ammonium acetate, 56 mM Tris-acetate pH 7.4, 1.2 mM ATP, 0.85 mM of GCU mix, 30 mM PEP, 5 %(w/v) PEG8000 and 2.0 mM (each) of the 20 amino acids. The addition of *T. kodakaraensis* tRNA was also tested, but there was no enhancement of ChiAΔ4 protein synthesis (data not shown).

Temperature preference.

Next, the effects of temperature on the system were examined. Western blot analysis revealed the synthesis of ChiAΔ4 within a temperature range from 40 °C to 75 °C, with a maximum at 65 °C (Fig. 3A). To examine whether the ChiAΔ4 protein was synthesized in an active form, the chitinase activity in the reaction mixture was measured. Significant levels of activity were detected within a temperature range of 40 °C to 80 °C, and highest activity was observed at 65 °C (Fig. 3B). This activity profile was consistent with the results of Western blot analysis, indicating that the ChiAΔ4 protein was most likely synthesized with the proper tertiary structure. Using the specific activity value of purified ChiAΔ4 expressed in *E. coli*, the maximum yield of active ChiAΔ4 was estimated to be approximately 1.3 μg/mL (at 65 °C).

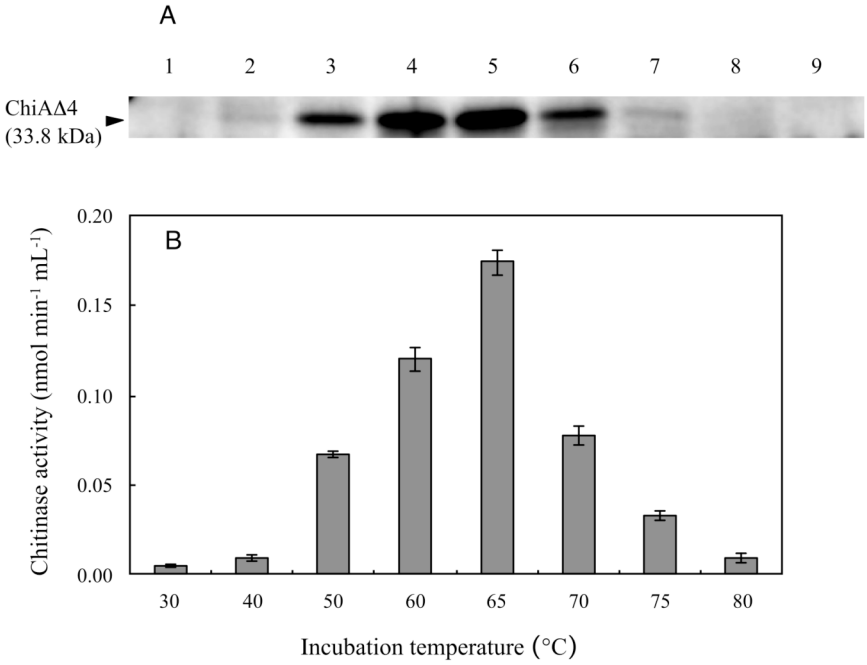

Fig. 3 Effect of temperature on cell-free protein synthesis with *T. kodakaraensis* S30 extract. (A) Each reaction mixture containing 0.4 mg/mL of ChiAΔ4 mRNA was incubated at 30, 40, 50, 60, 65, 70, 75 or 80 °C for 90 min (lanes 1-8, respectively). A negative control reaction was performed at 60 °C in the absence of mRNA (lane 9). ChiAΔ4 was visualized by Western blot analysis using rabbit anti-ChiAΔ4 antibodies. (B) Chitinase activity at various temperatures. Activity measurements were performed with and without the addition of substrate at each temperature, and the difference in values was calculated. Results are the average of n = 3 reactions and error bars represent standard deviations.

Time course of protein synthesis and determination of rate-limiting factors.

A time course experiment to monitor ChiAΔ4 protein levels showed that, at 65 °C, synthesis of ChiAΔ4 saturated at approximately 30 min (Fig. 4). At 70 °C, a rapid accumulation of ChiAΔ4 was observed in the first 5 min, and neared saturation at 15 min. A slower accumulation of ChiAΔ4 was observed at 60 °C, with protein synthesis continuing for over 60 min. On the other hand, no significant accumulation of ChiAΔ4 could be observed at 80 °C.

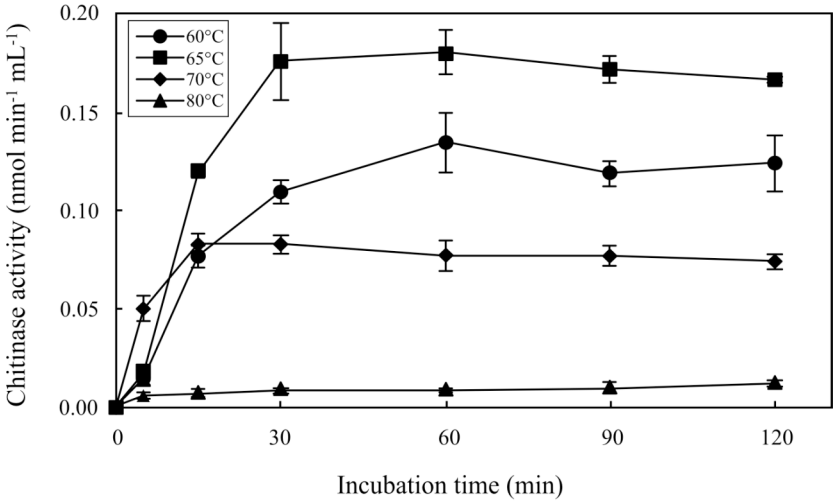

Fig. 4 Time course of cell-free protein synthesis with *T. kodakaraensis* S30 extract. Reactions were performed at 60 °C (circles), 65 °C (squares), 70 °C (diamonds) and 80 °C (triangles). Aliquots of sample were taken at 5, 15, 30, 60, 90 and 120 min after the initiation of the reaction and enzyme activities were measured. Results are the average of n = 3 reactions and error bars represent standard deviations.

The author examined the cause for the short duration of the reaction by performing semibatch reactions at the optimum temperature. After reactions were carried out for 45 min, mRNA, ATP and PEP were added individually. Figure 5 shows a time course of the accumulation of ChiAΔ4 protein in each reaction mixture. The addition of ATP and PEP had similar effects; the amount of ChiAΔ4 increased in the first 30 min and then leveled off. The addition of mRNA did not lead to enhanced synthesis. From this result, it appears that energy depletion is the main cause of the saturation in protein synthesis.

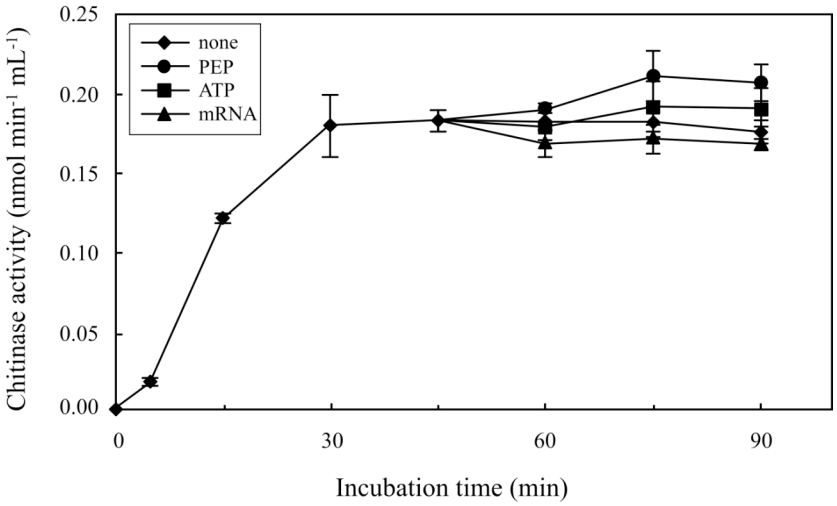

Fig. 5 Determination of rate-limiting factors. Reactions were performed in a total volume of 30 μl at 65°C for 45 min, and then one of the following components were added, none (diamonds), PEP (2.0 μmol, circles), ATP (36 nmol, squares) or ChiAΔ4 mRNA (12 μg, triangles), and incubation was continued for a further 45 min. Results are the average of n = 3 reactions and error bars represent standard deviations.

Transcription and translation-coupled protein synthesis.

Using the *T. kodakaraensis* S30 extract, the author also developed a coupled reaction system for cell-free transcription and translation at high temperatures. Instead of mRNA, the reaction mixture contained pTRC1 (Fig. 1) as a template DNA harboring a ChiAΔ4-encoding gene under the control of the T7 promoter, and thermostable T7 RNA polymerase. The reaction mixture was incubated at 40 °C for 1 h (for transcription), and then the temperature was shifted to 60 °C and incubation was continued for another 90 min (for translation). Using the two-step reaction, synthesis of active ChiAΔ4 could be detected. Increasing the levels of T7 RNA polymerase until 750 units led to higher amounts of synthesized protein, but further addition of the enzyme had a slightly negative effect (Fig. 6).

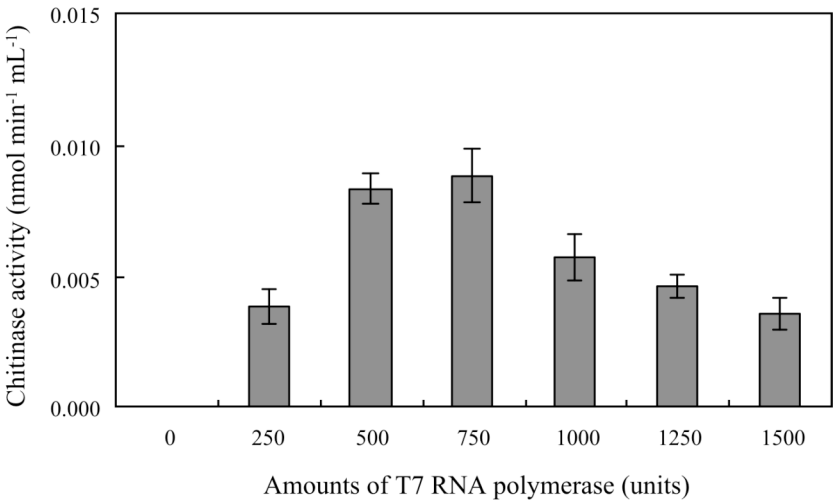

Fig. 6 Transcription and translation-coupled reaction. Reaction mixtures containing 2.0 μg of pTRC1 were first incubated at 40 °C for 60 min. Temperature was then shifted to 60 °C, and the reaction was further continued for 90 min. Activity measurements were performed with and without the addition of T7 RNA polymerase, and the difference in values was calculated. Results are the average of n = 3 reactions and error bars represent standard deviations.

DISCUSSION

The present study reports the development of a system for cell-free protein synthesis at high temperatures using *T. kodakaraensis* S30 extract. Synthesis of ChiAΔ4 was detected by Western blot analysis in a temperature range between 40 and 75 °C (Fig. 3A). ChiAΔ4 could not be detected by Western blot analysis at 80 °C, while chitinase activity at 80 °C was almost the same as that detected at 40 °C (Fig. 3B). The activity observed at 80 °C may be due to degradation products of ChiAΔ4 that still maintained enzymatic activity.

There was a significant difference between the optimal temperature of cell-free protein synthesis (65 °C) and the optimal growth temperature of *T. kodakaraensis* (85 °C). Further experiments are necessary to clearly explain the

difference, but the formation of precipitate after incubation of the reaction mixture at 85 °C may indicate that proteinous components in the S30 extract are undergoing thermal denaturation *in vitro*. In living-cells, induction of the chaperon system protects cellular proteins from thermal denaturation (11). It has been reported that low molecular substances such as trehalose have the ability to stabilize proteins in high temperature environments (1). Therefore, addition of such compounds to the reaction mixture might help to increase the optimum reaction temperature.

In this chapter, the author initially employed the reaction conditions and methods of Ellman et al. (5) for cell-free protein synthesis. As the author could not detect protein synthesis, the method of Pratt (20) was applied with some modifications, leading to favorable results. This is most likely due to the lower concentration of amino acids in the former system (0.35 mM for each amino acid) than in the latter system (2.0 mM). Indeed, the author observed a drastic decrease in protein production levels in the optimized system when amino acid concentrations were decreased (data not shown). On the other hand, several compounds in the Ellman method that were not present in our modified Pratt method (folic acid, pyridoxine hydrochloride, $NADP^+$, FAD, *p*-aminobenzonic acid and calcium ion) may have had an inhibitory effect against protein synthesis.

In the *T. kodakaraensis* system, ChiAΔ4 synthesis nearly saturated within 30 min of incubation at 65 °C, and the highest concentration of protein obtained was approximately 1.3 μg/mL. When compared with other systems, 100-fold higher protein concentrations have been obtained in 60 min using the optimized *E. coli* system (14, 15). Therefore, the speed of protein synthesis in the *T. kodakaraensis* system is, at least at present, considerably lower than the author had expected. Since the addition of energy-supplying substrates supported further synthesis of ChiAΔ4, energy depletion

can be regarded as the main cause of the arrest in protein synthesis. In general, high-energy compounds are unstable at high temperatures. The half-lives of ATP and PEP at high temperatures have been reported; the half-life of PEP is 20 min at 70 °C (27), while that of ATP is 115 min at 90 °C (in buffer containing Mg^{2+}) (13). It can be reasonably presumed that the half-lives of these compounds are even shorter in cell lysate, as reported in the *E. coli* system (14). A simple increase in the initial concentration of PEP (from 33 mM to 66 mM) was not effective, and rather had an inhibitory effect, probably due to the excess accumulation of inorganic phosphate (14). Developing a means to provide a stable supply of energy will be a key factor in increasing the production rate and overall yield of the system.

The use of hyperthermophiles as a source of cell lysate has various advantages. As the author has observed protein synthesis at temperatures as low as 40 °C, further optimization of the reaction conditions may allow application of the system for the synthesis of mesophilic proteins. A moderate elevation in temperature should lead to an increase in the production rate and may also provide an advantage in preventing inhibitory mRNA secondary structures. On the other hand, the system can also be utilized at higher temperatures ranging from 50 to 75 °C. This will provide a means to produce proteins from (hyper)thermophiles at temperatures near the native environment. When proteins from (hyper)thermophiles are synthesized in mesophiles, they are in many cases produced in a “semi-mature” form, exhibiting lower activity than that of the native protein. This is thought to be due to the entrapment of the protein molecule in an intermediary state of the folding process at low temperatures. This may be one of the main reasons why a number of proteins deriving from hyperthermophiles cannot be expressed in an active form in mesophilic hosts (22). The system developed in this chapter may provide an alternative in synthesizing these proteins in an active

form. Hyperthermophilic proteins synthesized in mesophilic hosts can be brought to their optimal, fully active states by incubating them at high temperature (22). Recombinant ChiAΔ4 synthesized in *E. coli* is one example, and the specific activity of the protein increases after an incubation of 10 min at 90 °C (data not shown). In contrast, the author observed that the specific activity of the ChiAΔ4 synthesized by the *in vitro* system at 65 °C did not change after heat treatment, indicating that the protein was produced in the optimal, thermostable form (data not shown).

The author also examined the possibilities of coupling a T7 RNA polymerase-dependent transcription reaction with his translation reaction with the *T. kodakaraensis* S30 extract. Initial attempts with a single reaction temperature did not lead to ChiAΔ4 synthesis at 40 °C, 50 °C or 60 °C (data not shown). This was most likely due to the difference in the optimum temperatures between the transcription and translation reactions: the temperature optimum of the *T. kodakaraensis* cell-free translation reaction is 65 °C, whereas the T7 RNA polymerase used has an optimum temperature of 50 °C, with only negligible activity above 55 °C. By utilizing a two-step system (reaction temperature was set at 40 °C and later changed to 60 °C), production of ChiAΔ4 was observed, and the optimal amount of T7 RNA polymerase was 750 units (in 30 μL of reaction mixture). The decrease in ChiAΔ4 synthesis with greater amounts of T7 RNA polymerase may be due to excess consumption of ATP in RNA synthesis, resulting in a shortage of ATP to be used for translation. Indeed, when the author decreased the amount of template DNA (0.6 μg), the apparent optimal amount of T7 RNA polymerase increased to 1,250 units (unpublished data). As DNA is much more stable than RNA and can be readily amplified by PCR, optimization of this coupled system will be an important subject to address in future studies.

Among hyperthermophiles, *T. kodakaraensis* is one of the few

microorganisms for which the entire genome sequence (7) and genetic transformation technology (24, 25) are both available. This unique feature of *T. kodakaraensis* will enable us to undertake molecular alteration of the species by removing genes encoding proteins that are disadvantageous for cell-free protein synthesis or by overexpressing genes that are favorable for the reaction.

REFERENCES

1. **Carninci, P., Y. Nishiyama, A. Westover, M. Itoh, S. Nagaoka, N. Sasaki, Y. Okazaki, M. Muramatsu, and Y. Hayashizaki.** 1998. Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. *Proc. Natl. Acad. Sci. USA* **95:**520-524.

2. **Chrunyk, B. A., J. Evans, J. Lillquist, P. Young, and R. Wetzel.** 1993. Inclusion body formation and protein stability in sequence variants of interleukin-1β. *J. Biol. Chem.* **268:**18053-18061.

3. **Condo, I., A. Ciammaruconi, D. Benelli, D. Ruggero, and P. Londei.** 1999. Cis-acting signals controlling translational initiation in the thermophilic archaeon *Sulfolobus solfataricus*. *Mol. Microbiol.* **34:**377-384.

4. **Dvorak, H. F., R. W. Brockman, and L. A. Heppel.** 1967. Purification and properties of two acid phosphatase fractions isolated from osmotic shock fluid of *Escherichia coli*. *Biochemistry* **6:**1743-1751.

5. **Ellman, J., D. Mendel, S. Anthony-Cahill, C. J. Noren, and P. G. Schultz.** 1991. Biosynthetic method for introducing unnatural amino acids site-specifically into proteins. *Methods Enzymol.* **202:**301-336.

6. **Endo, Y., and T. Sawasaki.** 2003. High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *Biotechnol.*

Adv. **21:**695-713.

7. **Fukui, T., H. Atomi, T. Kanai, R. Matsumi, S. Fujiwara, and T. Imanaka.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. *Genome Res.* **15:**352-363.

8. **Goff, S. A., and A. L. Goldberg.** 1987. An increased content of protease La, the *lon* gene product, increases protein degradation and blocks growth in *Escherichia coli*. *J. Biol. Chem.* **262:**4508-4515.

9. **Hempel, R., J. Schmidt-Brauns, M. Gebinoga, F. Wirsching, and A. Schwienhorst.** 2001. Cation radius effects on cell-free translation in rabbit reticulocyte lysate. *Biochem. Biophys. Res. Commun.* **283:**267-272.

10. **Henrich, B., W. Lubitz, and R. Plapp.** 1982. Lysis of *Escherichia coli* by induction of cloned ϕ X174 genes. *Mol. Gen. Genet.* **185:**493-497.

11. **Ideno, A., M. Furutani, Y. Iba, Y. Kurosawa, and T. Maruyama.** 2002. FK506 binding protein from the hyperthermophilic archaeon *Pyrococcus horikoshii* suppresses the aggregation of proteins in *Escherichia coli*. *Appl. Environ. Microbiol.* **68:**464-469.

12. **Kanai, T., H. Imanaka, A. Nakajima, K. Uwamori, Y. Omori, T. Fukui, H. Atomi, and T. Imanaka.** 2005. Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. *J. Biotechnol.* **116:**271-282.

13. **Kengen, S. W. M., A. J. M. Stams, and W. M. de Vos.** 1996. Sugar metabolism of hyperthermophiles. *FEMS Microbiol. Rev.* **18:**119-137.

14. **Kim, D. M., and J. R. Swartz.** 1999. Prolonging cell-free protein synthesis with a novel ATP regeneration system. *Biotechnol. Bioeng.* **66:**180-188.

15. **Kim, R. G., and C. Y. Choi.** 2001. Expression-independent consumption of substrates in cell-free expression system from *Escherichia coli*. *J. Biotechnol.* **84:**27-32.

16. **Marston, F. A.** 1986. The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **240:**1-12.

17. **Matthaei, H., and M. W. Nirenberg.** 1961. The dependence of cell-free protein synthesis in *E. coli* upon RNA prepared from ribosomes. *Biochem. Biophys. Res. Commun.* **4:**404-408.

18. **Myers, T. W., and D. H. Gelfand.** 1991. Reverse transcription and DNA amplification by a *Thermus thermophilus* DNA polymerase. *Biochemistry* **30:**7661-7666.

19. **Noren, C. J., S. J. Anthony-Cahill, M. C. Griffith, and P. G. Schultz.** 1989. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244:**182-188.

20. **Pratt, J. M.** 1984. Coupled transcription-translation in procaryotic cell-free systems, p. 179-207. *In* B. D. Hames and S. J. Higgins (ed.), Transcription and Translation; A Practical Approach. IRL Press, Oxford.

21. **Rahman, R. N. Z. A., S. Fujiwara, M. Takagi, and T. Imanaka.** 1998. Sequence analysis of glutamate dehydrogenase (GDH) from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1 and comparison of the enzymatic characteristics of native and recombinant GDHs. *Mol. Gen. Genet.* **257:**338-347.

22. **Rahman, R. N. Z. A., S. Fujiwara, M. Takagi, S. Kanaya, and T. Imanaka.** 1997. Effect of heat treatment on proper oligomeric structure formation of thermostable glutamate dehydrogenase from a hyperthermophilic archaeon. *Biochem. Biophys. Res. Commun.* **241:**646-652.

23. **Ruggero, D., R. Creti, and P. Londei.** 1993. In vitro translation of archaeal

natural mRNAs at high temperature. *FEMS Microbiol. Lett.* **107:**89-94.

24. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2005. Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. *Appl. Environ. Microbiol.* **71:**3889-3899.

25. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2003. Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J. Bacteriol.* **185:**210-220.

26. **Sato, T., H. Imanaka, N. Rashid, T. Fukui, H. Atomi, and T. Imanaka.** 2004. Genetic evidence identifying the true gluconeogenic fructose-1,6-bisphosphatase in *Thermococcus kodakaraensis* and other hyperthermophiles. *J. Bacteriol.* **186:**5799-5807.

27. **Schramm, A., B. Siebers, B. Tjaden, H. Brinkmann, and R. Hensel.** 2000. Pyruvate kinase of the hyperthermophilic crenarchaeote *Thermoproteus tenax*: physiological role and phylogenetic aspects. *J. Bacteriol.* **182:**2001-2009.

28. **Spirin, A. S., V. I. Baranov, L. A. Ryabova, S. Y. Ovodov, and Y. B. Alakhov.** 1988. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **242:**1162-1164.

29. **Tanaka, T., S. Fujiwara, S. Nishikori, T. Fukui, M. Takagi, and T. Imanaka.** 1999. A unique chitinase with dual active sites and triple substrate binding sites from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1. *Appl. Environ. Microbiol.* **65:**5338-5344.

30. **Tanaka, T., T. Fukui, and T. Imanaka.** 2001. Different cleavage specificities of the dual catalytic domains in chitinase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J. Biol. Chem.* **276:**35629-35635.

31. **Tulin, E. E., K. Tsutsumi, and S. Ejiri.** 1995. Continuously coupled transcription-translation system for the production of rice cytoplasmic aldolase. *Biotechnol. Bioeng.* **45:**511-516.

CHAPTER 2

A highly productive system for cell-free protein synthesis using a lysate of the hyperthermophilic archaeon, *Thermococcus kodakaraensis*

INTRODUCTION

A common methodology for cell-free protein synthesis is to use the lysates from various organisms. In particular, systems using *Escherichia coli* and wheat germ lysates have been developed greatly through improvements in lysate preparation (4, 14, 17), optimization of the reaction mixture composition (10, 11) or genetic alterations of host cells (8). As a result, over 300 μg/mL of productivity with the *E. coli* system (15, 29), and 55 μg/mL of productivity with the wheat germ system (19) were achieved in 1 hour of batch reaction.

As described in Chapter 1, the author was able to develop a novel system for cell-free protein synthesis that can be operated at high temperatures using a lysate of *Thermococcus kodakaraensis*. Using the system, synthesis of ChiAΔ4, a truncated form of *T. kodakaraensis* chitinase (25), was observed within a temperature range of 40 to 80 °C. Although cell-free protein synthesis was observed at an elevated temperature, the maximum yield of the synthesized protein remained at 1.3 μg/mL. In addition, there was a significant difference between the optimal growth temperature of *T. kodakaraensis* (85 °C) and the optimal temperature of cell-free protein synthesis (65 °C). Around the optimal growth temperature, the speed of protein synthesis inside of cells should be very rapid, and therefore, if the optimal reaction temperature of cell-free protein synthesis can be increased close to 85 °C, there is a possibility that the yield of protein synthesized might increase as a result of a higher speed of protein synthesis.

To improve the productivity of the *T. kodakaraensis* system, the author has examined the lysate preparation process as well as reaction mixture compositions. In addition, gene manipulation of *T. kodakaraensis* relating to an intracellular heat shock regulator was tested and its effect on cell-free protein synthesis was evaluated. As a result, over 100 μg/mL of productivity was achieved with the *T. kodakaraensis* system in a 15 min of batch reaction.

MATERIALS AND METHODS

Chemicals.

Sulfur, Tris-acetate, ammonium acetate, polyethyleneglycol 8000 (PEG8000), potassium phosphoenolpyruvate (PEP), trehalose, ectoin and hydroxyectoin were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP, GTP, CTP, UTP, 20 amino acids and betaine hydrochloride were from Sigma (St. Louis, MO, USA). RNase inhibitor was RNAsecure^{TM} from Ambion (Austin, TX, USA). All the other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

Construction of *T. kodakaraensis* Δ*phr* strain.

Disruption of *phr* gene (TK2291) by double-crossover homologous recombination was performed using the technique developed for *T. kodakaraensis* as described in previously (22, 23). The plasmid DNA used for disruption of *phr* was constructed as follows. A DNA fragment containing the *phr* coding region together with its flanking regions (about 1,000 bp) was amplified with the primer sets PKHR-L1 (5'-TGTCGTTCCAAAGCCAAAGG-3') and PKHR-R2 (5'-TGTCTCTCCCTCTTC CCTGG-3') using genomic DNA of *T. kodakaraensis* KOD1 as a template, and inserted into the HincII site of pUC118. Using the constructed plasmid DNA as a template, the

flanking regions of *phr* along with the plasmid backbone were amplified using primer sets PKHR-L2 (5'-CCCTTTCCCTAACCCAAAGT-3') and PKHR-R1 (5'-GAAGTCG TTAAAGGAGAAAG-3'), and the amplified fragment was designated as L-Phr. A PvuII-PvuII restriction fragment (763 bp) containing the *pyrF* marker gene was excised from pUD2 (22), and ligation was performed with L-Phr to construct the plasmid for *phr* disruption (pUPhr). A *T. kodakaraensis* uracil-auxotroph strain, KU216 (22), was used as a host strain for transformation, and *pyrF*$^{+}$ strain with uracil prototrophy was selected. The genotype of a Δ*phr* strain was confirmed by PCR amplification of a DNA fragment with a length corresponding to that of Δ*phr* locus (data not shown), and the constructed strain was named KHR1.

Preparation of *T. kodakaraensis* S30 extract.

T. kodakaraensis KC1 (Δ*chiA*) (see Chapter 1) and KHR1 (Δ*phr*) were precultured at 85 °C for about 12 h until A_{660} reached 0.2-0.4 in a nutrient-rich medium (MA-YT) (9) containing 0.5 %(w/v) elemental sulfur under anaerobic conditions. The preculture was used to inoculate 800 mL culture with MA-YT medium supplemented with 0.5 %(w/v) sodium pyruvate. This was cultured under anaerobic conditions at 85 °C until A_{660} reached 0.6-0.7 (about 14 h). Cells were harvested by centrifugation at 5,000 *g* for 10 min and washed two times with artificial sea water (0.8x Marine Art SF solution) (Tomita pharmaceuticals, Naruto, Japan) supplemented with 0.05 %(v/v) 2-mercaptoethanol.

The preparation of the S30 extract used for cell-free protein synthesis was performed based on the method described in Chapter 1, unless stated otherwise, under RNase-free conditions. Cells were suspended in S30 buffer (1.27 mL per gram of wet cells), which was composed of 10 mM Tris-acetate buffer (pH 7.4), 14 mM magnesium

acetate, 60 mM potassium acetate and 1.0 mM dithiothreitol (DTT). *T. kodakaraensis* cells were disrupted with a French press (FA-003, Thermo Electron Co., Waltham, MA, USA), and DTT was added to the resulting lysate to a final concentration of 1.0 mM (10 μL per mL of lysate). The lysate was then centrifuged at 30,000 *g* for 30 min at 4 °C. The upper four-fifths of the supernatant was collected, and a second 30,000 *g* centrifugation was repeated for 30 min at 4 °C, again collecting only the upper four-fifths of the supernatant. The mixture was then dialyzed three times (45 min each) against 40 times volume of S30 buffer using 7,000 MWCO dialysis tubes (Pierce Chemical, Rockford, IL, USA). After centrifugation at 4,000 *g* for 10 min, the resulting supernatant was used as S30 extract. Protein concentration was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. S30 extract was stored at -80 °C until use.

mRNA preparation.

mRNA encoding ChiAΔ4 was prepared with the T7 RiboMAX^{TM} Express RNA system (Promega, Madison, WI, USA) using a plasmid DNA, pTRC1 (see Chapter 1), treated with EcoRI as a template. The prepared mRNA was suspended in RNase-free water and stored at -80 °C until use.

Cell-free protein synthesis reaction.

The cell-free protein synthesis reaction was performed in a 30 μL batch scale using mRNA encoding ChiAΔ4 as a template. The reaction mixture contained ChiAΔ4 mRNA (0.4 mg/mL), *T. kodakaraensis* S30 extract made from either KC1 or KHR1, and other various ingredients shown in Table 1. The reaction was performed at 60 or 65 °C for 60 min, and then the reaction mixture was chilled on ice to stop reaction.

To improve productivity of the system, the method for preparing the S30 extract was first examined using *T. kodakaraensis* KC1. The modifications tested are listed in Table 2, Batches No. 1 to No. 3, which include modifications in the cell disruption, pre-incubation and dialysis steps. Translation activities of S30 extracts were examined at 65 °C for 60 min using the initial mixture composition shown in Table 1 (Batches No.1 to No. 3 in Table 2).

Next, with S30 extract prepared by the improved method, the reaction mixture composition was changed. The concentration of the *T. kodakaraensis* S30 extract was first varied (from 8.0 to 20 mg/mL) (Fig. 1A). Next, with 16 mg/mL S30 extract, concentrations of reaction constituents were changed within the ranges shown bellow: 0 to 10 mM magnesium acetate; 0 to 500 mM potassium acetate; 0 to 125 mM ammonium acetate; 0 to 25 mM PEP; 0 to 3 mM (each) 20 amino acid mixture (20AA mix); 0 to 6 %(w/v) PEG8000 (Fig. 1B-G, Fig. 2).

With the above optimized mixture composition (2^{nd} composition in Table1), yields of protein synthesis were compared between S30 extracts made from *T. kodakaraensis* KC1 and KHR1. Reactions were performed at 55 °C, 60 °C, 65 °C and 70 °C for 60 min (Fig. 3) (Batches No. 4 and No. 5 in Table 2).

Using S30 extract made from KHR1, concentrations of ATP and GCU mix (mixture of GTP, CTP and UTP) were changed within the ranges shown below; 0 to 7 mM ATP; 0 to 5 mM (each) GCU mix (Fig. 4AB). Initial pH value of Tris-Acetate buffer was also varied from 7.0 to 9.0 (Fig. 4C).

Finally, with the reaction mixture composition so far optimized (3^{rd} composition in Table 1) and S30 extract made from KHR1, the cell-free translation reaction was performed at 65 °C (Batch No. 6 in Table 2), and the reaction time course was monitored (Fig. 5).

Table 1. Reaction mixture compositions

Components	Unit	Initial composition[*1]	2nd composition	3rd composition
S30 extract	mg/mL	8.0	16	16
$Mg(OAc)_2$[*2]	mM	7.5	3.0	4.0
K(OAc)[*2]	mM	100	250	250
$NH_4(OAc)$[*2]	mM	80	80	80
Tris-Acetate (pH 7.4)	mM	56	56	-
Tris-Acetate (pH 8.2)	mM	-	-	56
ATP	mM	1.2	1.2	3.0
GCU mix[*3]	mM(each)	0.85	0.85	1.5
PEP[*4]	mM	30	10	10
PEG8000[*5]	%(w/v)	5.0	2.0	2.0
Spermidine	mM	-	-	0.2
20AA mix[*6]	mM(each)	2.0	2.0	2.0
mRNA	mg/mL	0.4	0.4	0.4
RNase inhibitor[*7]	%(v/v)	4.0	4.0	4.0

*1 The best mixture condition in Chapter 1

*2 OAc = Acetate

*3 GTP, CTP and UTP mixture

*4 Phosphoenolpyruvate

*5 Polyethyleneglycol 8000

*6 Mixture containing 20 amino acids

*7 RNAsecure™ (Ambion, Austin, TX) was used as a RNase inhibitor

Table 2. Process to search for the optimized reaction condition

Batch name	*T. kodakaraensis* strain	French press: Pressure (psi), Number of passes	Pre-incubation	Mixture composition[*1]	Reaction temperature	Maximum yield of ChiAΔ4[*2] (μg/mL)
Batch No. 1[*3]	KC1	10,000, 3 passes	Yes	Initial composition	65 °C	1.3±0.0
Batch No. 2	KC1	7,500, 1 pass	Yes	Initial composition	65 °C	2.0±0.2
Batch No. 3	KC1	7,500, 1 pass	No	Initial composition	65 °C	5.0±0.1
Batch No. 4	KC1	7,500, 1 pass	No	2nd composition	60 °C	74.9±4.0
Batch No. 5	KHR1	7,500, 1 pass	No	2nd composition	65 °C	85.0±2.5
Batch No. 6	KHR1	7,500, 1 pass	No	3rd composition	65 °C	115.4±4.8 [*4]

*1 See Table 1 for detail

*2 Protein yields after 60 min of reaction are shown except for Batch No. 6

*3 The best condition in Chapter 1

*4 Protein yield at 30 min of reaction is shown

Enzyme assay.

The chitinase activity assay was performed according to the procedure described in Chapter 1 using a fluorometric substrate, 4-methylumbelliferyl β–D-*N*,*N*'-diacetyl chitobioside (Sigma). After a 30 min reaction at 90 °C, the fluorescence of liberated 4-methylumbelliferone was measured (excitation: *ca*. 365 nm; emission: 460 nm) with a NanoDrop ND-3300 Fluorospectrometer (NanoDrop Technologies, Wilmington, NC, USA). Amount of the active ChiAΔ4 synthesized was calculated using the specific activity of purified ChiAΔ4 (0.135 nmol min^{-1} μg^{-1}).

Western blot analysis.

After cell-free protein synthesis, 0.1 μL of the reaction mixture was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5 % acrylamide concentration). Western blot analysis was performed according to the procedure described in Chapter 1 using polyclonal antibodies against ChiAΔ4.

RESULTS

Improvement of lysate preparation method.

The preparation method of cell lysate is a crucial step for cell-free protein synthesis, largely affecting yield of total protein synthesis (4, 14, 17). Therefore, the author examined the method of preparing the *T. kodakaraensis* cell lysate. Setup pressure of French press (2,500, 5,000, 7,500 and 10,000 psi) and the number of passes (one to three passes) were varied at first. In Chapter 1, three passes at 10,000 psi were employed (Batch No. 1 in Table 2). As a result, disruption of cells by a single pass with a pressure of 7,500 psi was the best condition, and the level of synthesized protein was increased by approximately 1.5-fold (comparison of Batch No. 1 and Batch No. 2 in

Table 2). The author also examined the omission of pre-incubation step, as it was recently reported that this step could be skipped in the lysate preparation of some *E. coli* strains (14). As a result, the level of synthesized protein was increased by approximately 2.5-fold (comparison of Batch No. 2 and Batch No. 3 in Table 2), indicating that the pre-incubation step was rather harmful to the *T. kodakaraensis* lysate. Finally, the effect of dialysis in the last step of lysate preparation was examined, but omission of the dialysis step slightly decreased the yield of protein synthesis (data not shown).

Optimization of reaction mixture composition I.

To achieve a high level of cell-free protein synthesis, an appropriate concentration of each reaction constituent should be determined beforehand (26). In the *T. kodakaraensis* system, desirable concentrations of each reaction constituent were described in Chapter 1. However, this needs more consideration, as a wide range of reaction conditions remain untested; for some components, only two conditions were tested. In addition to this, as the preparation method of the S30 extract had been changed, the author re-examined conditions of the optimal reaction composition.

It is reported that protein concentration of lysate has a significant influence on yield of protein synthesis in the *E. coli* system (20). Therefore, the author first varied protein concentration of *T. kodakaraensis* S30 extract. The yield of ChiAΔ4 synthesis increased with increasing the concentration from 8 mg/mL, and then saturated over 16 mg/mL (Fig. 1A).

Next, with 16 mg/mL of S30 extract concentration employed, suitable concentrations of several mixture ingredients were determined. A sharp peak was detected for magnesium acetate, potassium acetate, ammonium acetate and PEP, at a concentration of 3.0 mM (Fig. 1B), 250 mM (Fig. 1C), 75 mM (Fig. 1D) and 10 mM

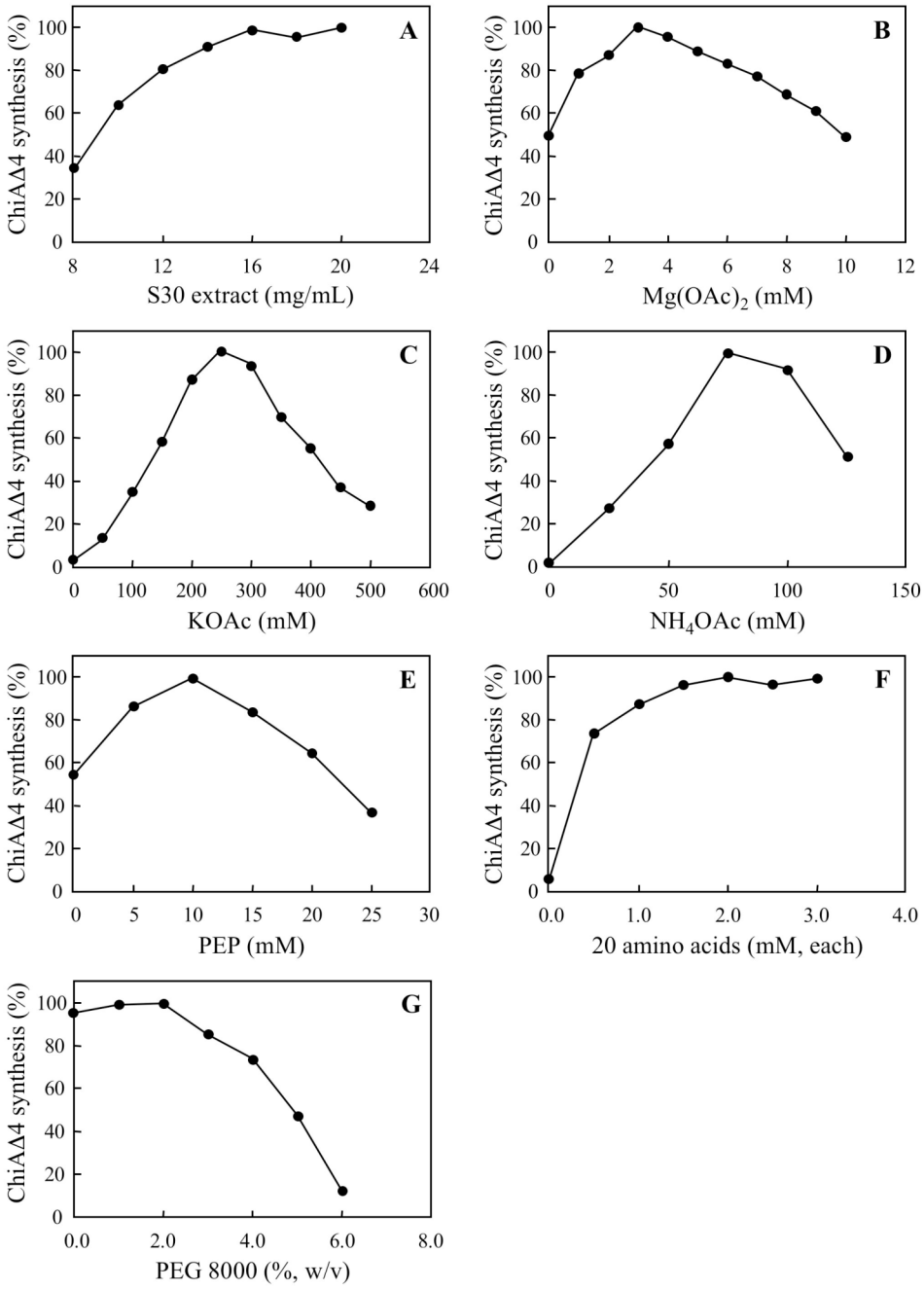

Fig. 1 Effect of various reaction constituents on the yield of cell-free protein synthesis using the *T. kodakaraensis* system. Reaction mixture containing 0.4 mg/mL of ChiAΔ4 mRNA was incubated at 65 °C for 90 min, and ChiAΔ4 synthesized was calculated from chitinase activity assay. Concentrations of the following constituents were varied: S30 extract (A), magnesium acetate (B), potassium acetate (C), ammonium acetate (D), PEP (E), 20AA mix (F), and PEG8000 (G). In each graph, the maximum value was set to 100 %.

(Fig. 1E), respectively. As for the 20 amino acids mixture (20AA mix), the yield of ChiAΔ4 synthesis increased until the 20AA mix concentration reached to 2.0 mM (each), and then leveled off (Fig. 1F).

Polyethyleneglycol, a common ingredient of the cell-free translation system, is regarded to contribute to the total yield of protein synthesis through stabilization of mRNA. In the *T. kodakaraensis* system, the optimal PEG8000 concentration was found to be 2.0 %(w/v), but over 90 % of activity was detected even in the absence of PEG8000 (Fig. 1G). However, when the synthesized protein was detected by Western blot analysis, a band that migrated slightly faster than ChiAΔ4 appeared in the PEG8000-free sample (Fig. 2A). The author think that this band corresponds to a readthrough product translated from the second methionine (Met^{13}) codon (ChiAΔ4-M13 in Fig. 2B), while retaining a comparable activity to the level of ChiAΔ4.

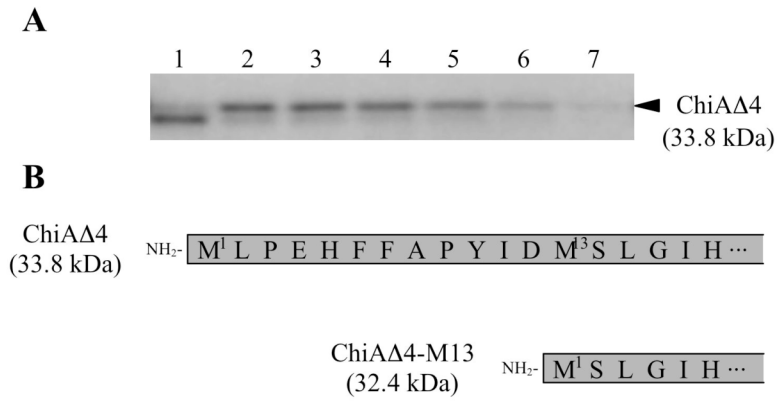

Fig. 2 (A) Effect of PEG8000 concentration on ChiAΔ4 synthesis. Reaction mixtures containing 0, 1, 2, 3, 4, 5 and 6 %(w/v) of PEG8000 were incubated at 65 °C for 90 min, and then separated on the 12.5 % SDS-PAGE gel (lanes 1–7, respectively). The protein synthesized was visualized by rabbit anti-ChiAΔ4 antibodies. (B) Proposed amino-terminal amino acid sequences and molecular weights of ChiAΔ4 and its derivative devoid of amino-terminal 12 amino acids (ChiAΔ4-M13).

The result here indicates that PEG8000 constitute an important component for producing ChiAΔ4 by the *T. kodakaraensis* system. Appropriate concentrations of reaction constituents shown in this section were summarized as the 2nd composition in Table 1.

As the author has changed preparation method of lysate and reaction mixture composition, temperature profile of reaction was re-examined in the 2nd mixture composition using S30 extract of *T. kodakaraensis* KC1. Within a range from 55 to 70 °C, the maximum yield was obtained at 60 °C (Fig. 3), which is a lower temperature than that described in Chapter 1 (65 °C). On the other hand, the yield of ChiAΔ4 synthesized in the condition notably increased to 74.9 μg/mL (Batch No. 4 in Table 2).

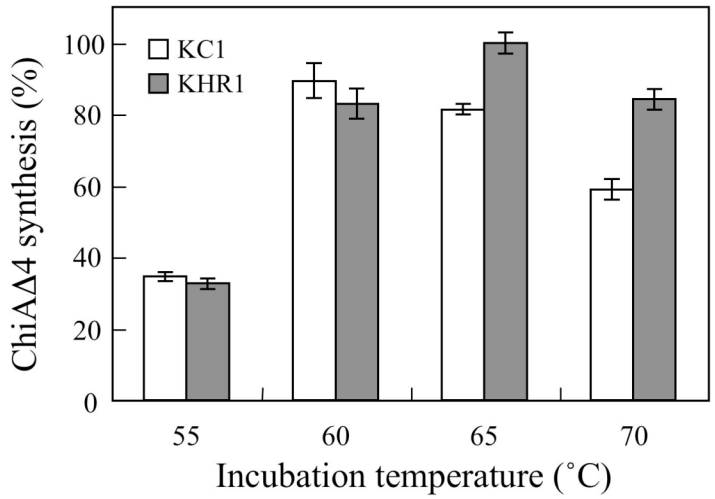

Fig. 3 Effect of reaction temperature on ChiAΔ4 synthesis was compared between S30 extracts made from *T. kodakaraensis* KC1 (open bars) and *T. kodakaraensis* KHR1 (filled bars). Reaction mixtures containing 0.4 mg/mL of ChiAΔ4 mRNA were incubated at 55, 60, 65 or 70 °C, for 60 min. Amount of ChiAΔ4 synthesized was estimated from chitinase activity assay. Results are the average of n = 3 reactions and error bars represent standard deviations. The maximum value was set to 100 %.

Attempts to increase the reaction temperature optimum.

The author next examined whether the reaction temperature optimum can be increased. In Chapter 1, a possibility was suggested that proteinous components in the S30 extract were undergoing thermal degradation over the optimal reaction temperature (see Chapter 1). Therefore, to increase the temperature optimum, it is important to prevent the lysate from thermal degradation.

In *Pyrococcus furiosus*, a close phylogenetic relative of *T. kodakaraensis*, there is a transcriptional factor, Phr, responsible for gene expressions of several heat shock proteins (HSPs) (27). A model is proposed that Phr binds to promoter regions of HSP genes and represses their transcriptions under normal growth temperature. Phr is conserved in several archaeal species and *T. kodakaraensis* contains its ortholog gene (TK2291). Here, the author hypothesized that the disruption of the *phr* gene would result in an increase in HSP levels in cells through derepression of HSP genes. If this is the case, use of the Δ*phr* strain as a source of S30 extract may result in an increase in the optimal reaction temperature. Therefore, Δ*phr* strain of *T. kodakaraensis* was constructed (strain KHR1), and S30 extract made from *T. kodakaraensis* KHR1 was used to examine the temperature profile of reaction. As a result, the maximum yield of ChiAΔ4 was observed at a higher temperature of 65 °C (Fig. 3). The yield of ChiAΔ4 synthesized in this condition was 85.0 μg/mL (Batch No. 5 in Table 2), which was significantly higher than the maximum yield using a lysate of KC1 (Batch No. 4 in Table 2).

It was reported that some low molecular substances, known as “compatible solute”, have a function to protect enzymes against heat inactivation (2, 3, 21). Therefore, the author also examined the effects of several compatible solutes added to the *T. kodakaraensis* system. While addition of trehalose (50 mM) virtually gave no

effect to the yield of protein synthesis at 65 °C, its addition at 67 °C and 69 °C resulted in 10-25 % increase in yields of protein synthesis. However, yields of protein production observed at 67 °C and 69 °C were still lower than that at 65 °C (data not shown). On the other hand, no considerable effects were found by the addition of ectoin (50 mM) or hydroxyectoin (50 mM) at 67 °C, while the addition of betaine (50 mM) completely inhibited protein synthesis at 67 °C.

Optimization of reaction mixture composition II.

Using *T. kodakaraensis* KHR1 as a source of S30 extract, concentrations of reaction constituents that were not verified in Chapter 1 were changed. Both ATP and GTP are the essential factors for translation reaction (26). The optimal concentrations of ATP and GCU mix were 3.0 mM (Fig. 4A) and 1.5 mM each (Fig. 4B), respectively. It is well known that all nucleoside triphosphates (NTP) are present as an NTP-Mg^{2+} complex in the cell (1). Followed by an increase in NTP concentration, the optimum concentration of magnesium acetate was changed to 4.0 mM.

Addition of spermidine was reported to enhance cell-free protein synthesis in the *E. coli* (28) and the insect (26) systems. The author also observed that addition of 0.2 mM spermidine was effective for the *T. kodakaraensis* system to some extent (about 5 %). Finally, initial pH value of Tris-Acetate buffer was examined and the optimal value was determined to be 8.2 (Fig. 4C). The best concentrations or value of reaction constituents shown in this section were summarized as the "3rd composition" in Table 1.

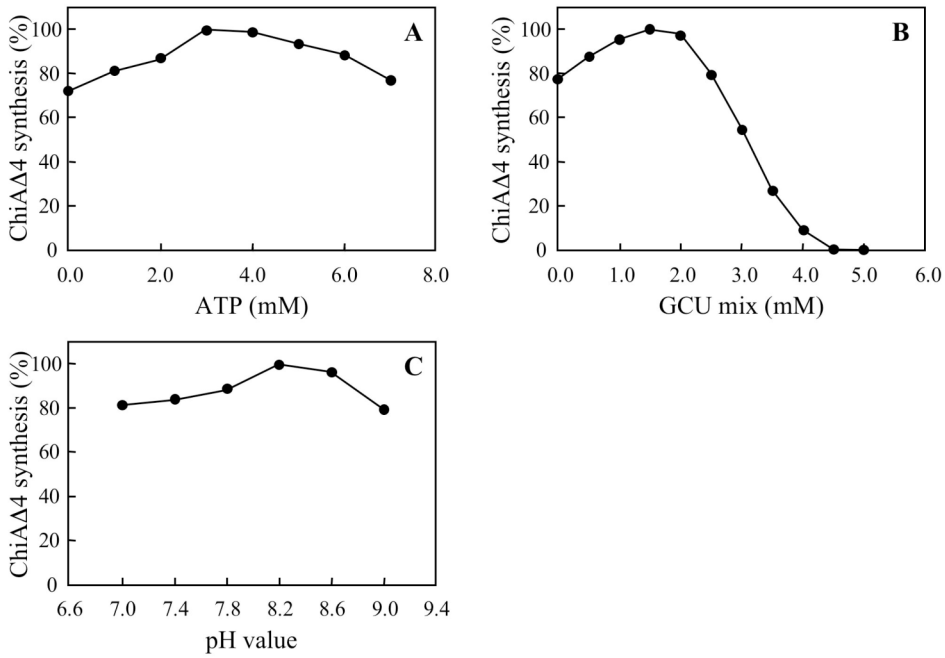

Fig. 4 Effect of various reaction constituents on the yield of cell-free protein synthesis using the *T. kodakaraensis* system. Reaction mixture containing 0.4 mg/mL of ChiAΔ4 mRNA was incubated at 65 °C for 90 min, and ChiAΔ4 synthesized was calculated by chitinase activity assay. Concentrations or values of the following constituents were varied: ATP (A), GCU mix (B) and initial pH value of Tris-Acetate buffer (C). In each graph, the maximum value was set to 100 %.

Reaction time course and yield of protein synthesis.

As an overall summary of the present study, cell-free protein synthesis was performed under the best reaction condition (Batch No. 6 in Table 2), and reaction time course was monitored by measuring chitinase activity. The amount of ChiAΔ4 synthesized increased rapidly, exceeding over 100 μg/mL within 15 min of incubation, and was nearly saturated at 30 min (Fig. 5A). The highest concentration of ChiAΔ4 obtained was 115.4 μg/mL (at 30 min), which is about 90 times higher than that in Chapter 1.

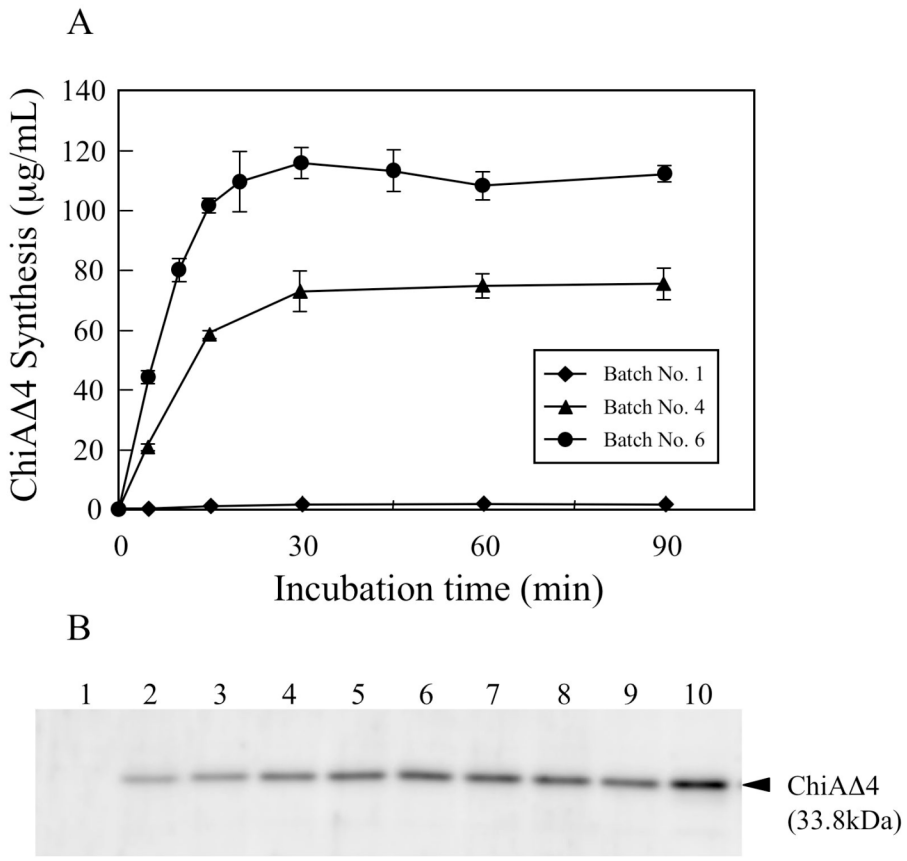

Fig. 5 Reaction time course of ChiAΔ4 synthesis. (A) Detection by activity measurement. Batch reactions were carried out under three different conditions: Batch No. 1 (filled diamonds), Batch No. 4 (filled triangles) and Batch No. 6 (filled circles). Detailed reaction conditions are described in Table 2. Reaction mixtures containing 0.4 mg/mL of ChiAΔ4 mRNA were incubated at 60 or 65 °C for up to 90 min, and the ChiAΔ4 synthesized was calculated by chitinase activity assay. Results are the average of n = 3 reactions and error bars represent standard deviations. (B) Detection by Western blot analysis. Reaction mixtures (Batch No. 6) incubated at 65 °C for 0, 5, 10, 15, 20, 30, 45, 60 or 90 min (lanes 1-9, respectively), were separated on the 12.5 % SDS-PAGE gel and the synthesized protein was visualized by rabbit anti-ChiAΔ4 antibodies. Purified recombinant ChiAΔ4 with a concentration of 200 μg/mL was applied as a positive control (lane 10).

To confirm the high productivity of the system, the ChiAΔ4 synthesized was detected by Western blot analysis (Fig. 5B), and signal intensities were quantified by a chemiluminescence image analyzer. Reaction samples corresponding to incubation times of 20, 30, 45, 60 and 90 min (lanes 5–9) gave about 60 to 68 % signal intensities of that of a positive control (lane 10). As the control fraction contained recombinant ChiAΔ4 with a concentration of 200 μg/mL, the data demonstrated that over 100 μg/mL ChiAΔ4 protein was actually produced by the *T. kodakaraensis*-based cell-free system.

DISCUSSION

As described in Chapter 1, the highest concentration of the synthesized protein (ChiAΔ4) by the *T. kodakaraensis* system was only 1.3 μg/mL, which was less than one hundredth of the optimized *E. coli* system (12, 13). In this study, through improvements in lysate preparation and reaction mixture compositions, as well as genetic modification of *T. kodakaraensis*, the protein yield of the *T. kodakaraensis* system was increased to 115.4 μg/mL in 30 min of batch reaction.

In the preparation of *E. coli* S30 extract, pre-incubation (runoff) reaction plays an important role to obtain a lysate with high translation activity by terminating ongoing translation reactions (17). In the lysate preparation described in Chapter 1, pre-incubation was performed at 37 °C, a relatively low temperature for *T. kodakaraensis*. However, an elevation of pre-incubation temperature to 60 °C resulted in a dramatic loss of translation activity (unpublished result). On the contrary, omitting the pre-incubation step rather increased the translation activity by 2.5-fold. At present, it is not clear why pre-incubation step is harmful for the *T. kodakaraensis* lysate, but the author think that the translational activity of the cell lysate might be lost after incubation at high temperature. Another possibility is that, as *T. kodakaraensis* is an obligate

anaerobe, a long-time exposure to air caused oxidation of the lysate, which might gave the fatal effect to the translational activity.

The speed of cell-free protein synthesis reported in a batch reaction of the *E. coli* system was over 300 μg mL^{-1} h^{-1} (15, 29). In the optimized *T. kodakaraensis* system described here, the highest speed of protein synthesis obtained was approximately 100 μg/mL in 15 min (Batch No. 6 in Fig. 5A), which is comparable to the levels of the *E. coli* system. However, a relatively short duration of reaction in the *T. kodakaraensis* system (about 30 min) hampers further increase in protein yield. In the *T. kodakaraensis* system, PEP is added as an energy substrate for ATP regeneration, but it was reported that PEP is easy to be degraded, especially in cell lysate (12). Recently, in the *E. coli* system, adoption of a new energy regeneration system composed of creatine kinase and creatine phosphate significantly contributed to prolong reaction time (11). Although the same system cannot be applied to the *T. kodakaraensis* system that works under high temperatures, developing a means to provide a stable supply of energy will be a key factor in increasing the overall yield of the system.

In the present study, the author has determined suitable concentrations of each reaction constituent (the best condition is shown in Table 1 as the 3rd composition). Among them, a high concentration of potassium ion (250 mM) is characteristic to the *T. kodakaraensis* system comparing to the *E. coli* system, in which about 100 mM concentration is normally employed (14). The preference for potassium ion may reflect a high intracellular potassium concentration of (hyper)thermophiles (6, 24). The requirement of a high potassium concentration (250 to 400 mM) was also reported in the cell-free transcription system operated at 90 °C using the *P. furiosus* RNA polymerase (7). The necessity of high potassium concentration in these systems may be explained by contribution of potassium ion to the thermostability of biological

molecules, such as DNA (18) and proteins (21).

T. kodakaraensis is one of the few microorganisms for which the entire genome sequence (5) and genetic transformation technology (22, 23) are both available among hyperthermophiles. In this study, the author have utilized these features to construct a gene disruption mutant (Δ*phr*), and an increase in the temperature optimum as well as increase in the yield of protein synthesis was achieved by using S30 extract made from the mutant. As Phr is suggested to be a transcriptional regulator repressing the HSP expression in normal growth temperature (27), it is proposed that intracellular levels of HSP in the Δ*phr* strain are significantly high. Actually, the author found that several proteins, including small heat shock protein, are induced in the Δ*phr* strain (unpublished data). It is very interesting that induction of a small numbers of proteins caused an increase in the optimum temperature of cell-free translation, a complex process in which numbers of reactions are concerned. This situation is very similar to a report showing that overexpression of small heat shock protein of a hyperthermophile in *E. coli* significantly increased the tolerance of cells against heat stress (16). The present result of an increase in the temperature optimum using the Δ*phr* lysate suggested that proteinous substrates are actually undergoing heat inactivation. Therefore, developing a way to prevent heat inactivation of lysate will also be an important topic to prolong reaction duration and thereby to increase the overall yield of the system.

REFERENCES

1. **Berg, J. M., J. L. Tymoczko, and L. Stryer.** 2002. Biochemistry 5th ed. W. H. Freeman and Co., New York.

2. **Borges, N., A. Ramos, N. D. Raven, R. J. Sharp, and H. Santos.** 2002. Comparative study of the thermostabilizing properties of mannosylglycerate and other

compatible solutes on model enzymes. *Extremophiles* **6:**209-216.

3. **Carninci, P., Y. Nishiyama, A. Westover, M. Itoh, S. Nagaoka, N. Sasaki, Y. Okazaki, M. Muramatsu, and Y. Hayashizaki.** 1998. Thermostabilization and thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. *Proc. Natl. Acad. Sci. USA* **95:**520-524.

4. **Endo, Y., and T. Sawasaki.** 2003. High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *Biotechnol. Adv.* **21:**695-713.

5. **Fukui, T., H. Atomi, T. Kanai, R. Matsumi, S. Fujiwara, and T. Imanaka.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. *Genome Res.* **15:**352-363.

6. **Grayling, R. A., K. Sandman, and J. N. Reeve.** 1996. DNA stability and DNA binding proteins, p. 437-467. *In* M. W. W. Adams (ed.), Enzyme and proteins from hyperthermophilic mmicroorganisms. Academic Press, California Tokyo.

7. **Hethke, C., A. Bergerat, W. Hausner, P. Forterre, and M. Thomm.** 1999. Cell-free transcription at 95°: thermostability of transcriptional components and DNA topology requirements of *Pyrococcus* transcription. *Genetics* **152:**1325-1333.

8. **Jiang, X., K. Oohira, Y. Iwasaki, H. Nakano, S. Ichihara, and T. Yamane.** 2002. Reduction of protein degradation by use of protease-deficient mutants in cell-free protein synthesis system of *Escherichia coli*. *J. Biosci. Bioeng.* **93:**151-156.

9. **Kanai, T., H. Imanaka, A. Nakajima, K. Uwamori, Y. Omori, T. Fukui, H. Atomi, and T. Imanaka.** 2005. Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. *J. Biotechnol.* **116:**271-282.

10. **Kawarasaki, Y., T. Kawai, H. Nakano, and T. Yamane.** 1995. A long-lived batch reaction system of cell-free protein synthesis. *Anal. Biochem.* **226:**320-324.

11. **Kigawa, T., T. Yabuki, Y. Yoshida, M. Tsutsui, Y. Ito, T. Shibata, and S. Yokoyama.** 1999. Cell-free production and stable-isotope labeling of milligram quantities of proteins. *FEBS Lett.* **442:**15-19.

12. **Kim, D. M., and J. R. Swartz.** 1999. Prolonging cell-free protein synthesis with a novel ATP regeneration system. *Biotechnol. Bioeng.* **66:**180-188.

13. **Kim, R. G., and C. Y. Choi.** 2000. Expression-independent consumption of substrates in cell-free expression system from *Escherichia coli*. *J. Biotechnol.* **84:**27-32.

14. **Kim, T. W., J. W. Keum, I. S. Oh, C. Y. Choi, C. G. Park, and D. M. Kim.** 2006. Simple procedures for the construction of a robust and cost-effective cell-free protein synthesis system. *J. Biotechnol.* **126:**554-561.

15. **Kim, T. W., D. M. Kim, and C. Y. Choi.** 2006. Rapid production of milligram quantities of proteins in a batch cell-free protein synthesis system. *J. Biotechnol.* **124:**373-380.

16. **Laksanalamai, P., D. L. Maeder, and F. T. Robb.** 2001. Regulation and mechanism of action of the small heat shock protein from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J. Bacteriol.* **183:**5198-5202.

17. **Liu, D. V., J. F. Zawada, and J. R. Swartz.** 2005. Streamlining *Escherichia coli* S30 extract preparation for economical cell-free protein synthesis. *Biotechnol. Prog.* **21:**460-465.

18. **Marguet, E., and P. Forterre.** 1998. Protection of DNA by salts against thermodegradation at temperatures typical for hyperthermophiles. *Extremophiles* **2:**115-122.

19. **Nakano, H., T. Tanaka, Y. Kawarasaki, and T. Yamane.** 1996. Highly

productive cell-free protein synthesis system using condensed wheat-germ extract. *J. Biotechnol.* **46:**275-282.

20. **Pratt, J. M.** 1984. Coupled transcription-translation in procaryotic cell-free systems, p. 179-207. *In* B. D. Hames and S. J. Higgins (ed.), Transcription and Translation; A Practical Approach. IRL Press, Oxford.

21. **Ramos, A., N. Raven, R. J. Sharp, S. Bartolucci, M. Rossi, R. Cannio, J. Lebbink, J. van der Oost, W. M. de Vos, and H. Santos.** 1997. Stabilization of enzymes against thermal stress and freeze-drying by mannosylglycerate. *Appl. Environ. Microbiol.* **63:**4020-4025.

22. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2005. Improved and versatile transformation system allowing multiple genetic manipulations of the hyperthermophilic archaeon *Thermococcus kodakaraensis*. *Appl. Environ. Microbiol.* **71:**3889-3899.

23. **Sato, T., T. Fukui, H. Atomi, and T. Imanaka.** 2003. Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J. Bacteriol.* **185:**210-220.

24. **Scholz, S., J. Sonnenbichler, W. Schäfer, and R. Hensel.** 1992. Di-myo-inositol-1,1'-phosphate: a new inositol phosphate isolated from *Pyrococcus woesei*. *FEBS Lett.* **306:**239-242.

25. **Tanaka, T., S. Fujiwara, S. Nishikori, T. Fukui, M. Takagi, and T. Imanaka.** 1999. A unique chitinase with dual active sites and triple substrate binding sites from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1. *Appl. Environ. Microbiol.* **65:**5338-5344.

26. **Tarui, H., M. Murata, I. Tani, S. Imanishi, S. Nishikawa, and T. Hara.** 2001. Establishment and characterization of cell-free translation/glycosylation in insect

cell (*Spodoptera frugiperda* 21) extract prepared with high pressure treatment. *Appl. Microbiol. Biotechnol.* **55:**446-453.

27. **Vierke, G., A. Engelmann, C. Hebbeln, and M. Thomm.** 2003. A novel archaeal transcriptional regulator of heat shock response. *J. Biol. Chem.* **278:**18-26.

28. **Yin, G., and J. R. Swartz.** 2004. Enhancing multiple disulfide bonded protein folding in a cell-free system. *Biotechnol. Bioeng.* **86:**188-195.

29. **Zawada, J., and J. R. Swartz.** 2006. Effects of growth rate on cell extract performance in cell-free protein synthesis. *Biotechnol. Bioeng.* **94:**618-624.

PART II

Application of the *T. kodakaraensis* cell-free protein synthesis system.

CHAPTER 3

Effective approaches for the production of heterologous proteins using the *Thermococcus kodakaraensis*-based translation system

INTRODUCTION

As described in Chapter 1 and 2, the author has developed a system for cell-free protein synthesis that can be operated at high temperatures using a lysate of *Thermococcus kodakaraensis*. To put this system to practical use in the field of protein production, it is necessary to synthesize proteins from organisms other than *T. kodakaraensis*. Therefore, the author attempted to synthesize green fluorescent protein (GFP), a protein from the jellyfish, *Aequorea victoria* (3), using the *T. kodakaraensis* cell-free system. Gene encoding GFP is frequently used as a reporter of expression, as GFP can be easily detected by its intrinsic fluorescence. As such, GFP is used as a reporter protein also in the field of cell-free protein synthesis (9, 12, 14). As the *T. kodakaraensis* system is operated under high temperature conditions, a thermostable GFP derivative (tGFP) was selected as a target protein.

In this chapter, the author examined three points that would be effective for the synthesis of active tGFP by the *T. kodakaraensis* cell-free system. These are as follows: 1) addition of stem-loop structure at the 3'-end of mRNA to prevent its nucleolytic degradation, 2) optimization of codon usage to improve the translational efficiency, and 3) addition of chaperonin oligomers to improve the protein folding efficiency. The positive effects brought about by these modifications towards the synthesis of tGFP provide valuable information for future synthesis of heterologous proteins using the *T. kodakaraensis* cell-free system.

MATERIALS AND METHODS

Chemicals.

Sulfur, Tris-acetate, ammonium acetate, polyethyleneglycol 8000, potassium phosphoenolpyruvate and spermidine were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP, GTP, CTP, UTP, and 20 amino acids were from Sigma (St. Louis, MO, USA). RNase inhibitor, RNAsecure^{TM}, was from Ambion (Austin, TX, USA). All the other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

Plasmid construction.

Gene encoding tGFP used in this study was derived from pET-GFP$_{(FRET)}$His (13), which encodes a GFP mutant with a red-shifted excitation peak and an improved folding efficiency. Amino acid sequence of the tGFP was deduced as MASKG. . .F64L/S65C/Q80R/F99S/M153T/V163A. . .THGSGGRLEHHHHHH. The underlined region indicates the amino acid sequence derived from GFP. Amino acid residues different from those of the wild-type GFP are indicated. Note that the amino acid numbers correspond to those in the wild-type GFP.

pTRG1, a plasmid used to prepare mRNA, was constructed as follows. A 755 bp-DNA fragment containing the tGFP gene was amplified from pET-GFP$_{(FRET)}$His by PCR using the following two primers, GFP-N1 (5'-AAAACATATGGCTAGCAAAGG AGAAGAACTCTTCA-3', underlined sequences correspond to NdeI site) and GFP-C1 (5'-AAAAGAATTCTCAGTGGTGGTGGTGGTGGTGCTCG-3', underlined sequences correspond to EcoRI site). After treatment with NdeI and EcoRI, the amplified fragment was inserted into the respective sites of pT2 (see Chapter 1), which contained T7 promoter and ribosome-binding site of the *T. kodakaraensis* glutamate dehydrogenase gene, to make pTRG1.

Gene coding for tGFP with codons optimized for *T. kodakaraensis* was constructed by referring to the Stemmer's method of single-step overlap extension PCR (15). Oligonucleotides were designed by referring to the DNAWorks web site (http://helixweb.nih.gov/dnaworks/) (6). Twenty-two oligonucleotides with the overlapping regions within 12–17 bases were prepared. The synthesized tGFP gene was arranged to have NdeI and EcoRI sites upstream of the initiation codon and downstream of the stop codon, respectively. After a reaction of overlap extension PCR, reaction products were treated with NdeI and EcoRI, and inserted into the respective sites of pT2, to make pTRG2 (Fig. 3A).

Nucleotide sequences that are able to form RNA secondary structures were inserted at the 3'-end of tGFP gene as follows. A 61 bp-DNA fragment containing an artificial stem-loop structure, pHP10, was synthesized using the following two primers, ChiA-SLN (5'- AAAAGGATCCATGCCATGGCAGTTGAATAGAGTGAGCT -3', underlined sequences correspond to BamHI site) and ChiA-SLC (5'-AAAAGAATTCTGCAGCTGAATAGAGCTCACTCT -3', underlined sequences correspond to EcoRI site). A 374 bp-DNA fragment containing the terminator region of *T. kodakaraensis* chitinase gene (*chiA*) was amplified by PCR with a plasmid DNA, pTRC1 (see Chapter 1), using the following two primers, ChiA-TN (5'-AAAAGGATCCCCTCTCTTCTCCTCTT-3', underlined sequences correspond to BamHI site) and ChiA-TC (5'-AAAAGAATTCAGCTGAAGAACAGGCTGAGG-3', underlined sequences correspond to EcoRI site). These DNA fragments were inserted, after treatment with BamHI and EcoRI, into the respective sites of pTRG2, to make pTRG2-SL (containing artificial stem-loop structure) and pTRG2-CT (containing the *chiA* terminator region) (Fig. 3A).

Preparation of recombinant tGFP, CpkA and CpkB.

Recombinant tGFP was expressed in *Escherichia coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene, La Jolla, CA, USA) harboring plasmid, pET-$GFP_{(FRET)}$His. The expressed tGFP with a poly-histidine tag at the carboxy-terminus was purified with the QuickPick^{TM} IMAC Plus kit (Bio-Nobile, Turku, Finland). Recombinant chaperonin oligomers were expressed in *E. coli* BL21-CodonPlus(DE3)-RIL cells harboring one of either plasmid, pCPAE (for CpkA expression) and pECPK (for CpkB expression) (8). Purifications of CpkA and CpkB oligomers were performed by the method described previously (8, 16).

mRNA preparation.

mRNA encoding tGFP was prepared with the T7 RiboMAX^{TM} Express RNA system (Promega, Madison, WI, USA) using a plasmid DNA (pTRG1, pTRG2, pTRG2-SL or pTRG2-CT) treated with EcoRI as a template. The prepared mRNA was suspended in RNase-free water and stored at -80 °C until use.

Reaction conditions for cell-free protein synthesis.

T. kodakaraensis lyasate used for cell-free protein synthesis (S30 extract) was prepared as described method in Chapter 2 using *T. kodakaraensis* KHR1 strain (see Chapter 2). Protein synthesis reactions were performed in a 30 μL mixture containing mRNA, *T. kodakaraensis* S30 extract (16 mg/mL), magnesium acetate (4.0 mM), potassium acetate (250 mM), ammonium acetate (80 mM), Tris-acetate (56 mM, pH 8.2), ATP (3.0 mM), GTP (1.5 mM), CTP (1.5 mM), UTP (1.5 mM), potassium phosphoenolpyruvate (10 mM), polyethyleneglycol 8000 (2 %, w/v), spermidine (0.2 mM), 20 amino acids (2.0 mM, each), and RNase inhibitor (RNAsecure^{TM}) (4.0 %, v/v).

These reaction mixtures were kept at a constant temperature for 60 or 90 min. In the experiments to examine the effect of *T. kodakaraensis* chaperonins, concentrations of ATP and magnesium acetate increased to 4.0 mM and 5.0 mM, respectively.

Western blot analysis.

After cell-free protein synthesis, reaction mixture corresponding to 0.25 μL was applied to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5 % acrylamide concentration). Western blot analysis was performed according to the procedure described in Chapter 1 using rabbit polyclonal antibodies against tGFP.

Fluorescence measurement of tGFP.

Fluorescence of tGFP was measured (excitation: *ca*. 470 nm; emission: 511 nm) with a NanoDrop ND-3300 Fluorospectrometer (NanoDrop Technologies, Wilmington, NC, USA). Amount of the fluorescence-active tGFP synthesized was calculated according to a calibration curve made using purified recombinant tGFP.

Effect of temperature on the stability of recombinant tGFP.

Recombinant tGFP (100 μg/mL) was added in a buffer used for cell-free protein synthesis in the presence or absence of *T. kodakaraensis* S30 extract. These mixtures were incubated at 60 or 70 °C for 30-90 min, and then cooled on ice. The remaining fluorescence was measured by NanoDrop ND-3300 Fluorospectrometer.

RESULTS

Stability test of recombinant tGFP.

As described in Chapter 2, the optimal reaction temperature of the *T. kodakaraensis*-based translation system is 60-65 °C, proteins to be synthesized by the system are required to have enough stability at the given reaction temperatures. Therefore, recombinant tGFP produced in *E. coli* was tested for its stabilities at 60 °C and 70 °C (Fig. 1). Purified tGFP was added in reaction mixtures for cell-free protein synthesis, and after 90 min of incubation at 60 °C, over 95 % of fluorescence was detected (Fig. 1A). Even at 70 °C, over 75 % of fluorescence remained (Fig. 1B), showing the high stability of tGFP under high temperature reaction conditions.

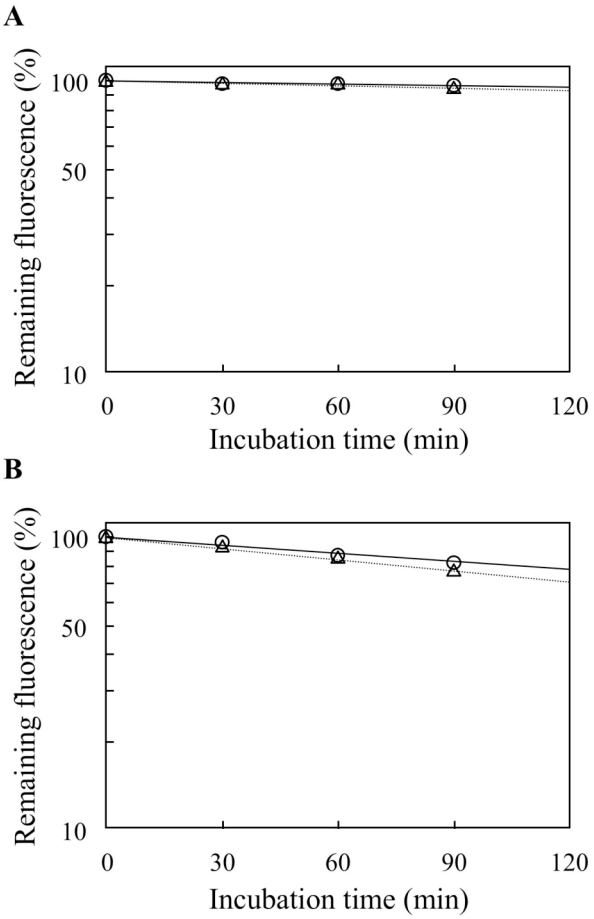

Fig. 1 Effect of temperature on the stability of recombinant tGFP. Recombinant tGFP with a concentration of 100 μg/mL was incubated in reaction mixture for cell-free protein synthesis in the presence (open triangle) or absence (open circle) of the *T. kodakaraensis* S30 extract. These mixtures were incubated at 60 °C (A) or 70 °C (B), and fluorescences of these mixtures were measured at the time indicated.

Cell-free synthesis of tGFP using a codon-optimized gene.

The author examined whether tGFP can be synthesized at 60 °C using the *T. kodakaraensis* S30 extract and mRNA prepared from a template, pTRG1. As a result, only a faint band corresponding to the molecular weight of tGFP was detected by Western blot analysis (Fig. 2A, lane 1). As the tGFP gene in pTRG1 was derived from an expression vector for *E. coli*, the author thought that difference in codon usage might

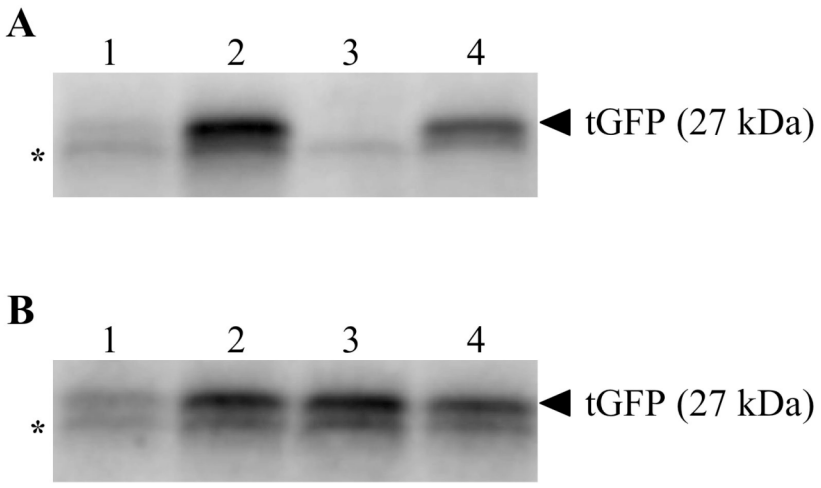

Fig. 2 Detection of tGFP by Western blot analysis. (A) Effect of codon alteration on tGFP synthesis. Reaction mixtures containing tGFP mRNA derived from pTRG1 (0.4 mg/mL, lane 1), tGFP mRNA derived from pTRG2 (0.4 mg/mL, lane 2) or no mRNA (lane 3) were incubated at 60 °C for 90 min. As a positive control, purified recombinant tGFP added at a concentration of 2.0 μg/mL in a reaction mixture of cell-free protein synthesis, was also applied (lane 4). The tGFP synthesized was detected using polyclonal anti-tGFP antibodies. Asterisk indicates a protein band reacted nonspecifically with anti-tGFP antibodies. (B) Effect of reaction temperatures on tGFP synthesis. Reaction mixtures containing 0.4 mg/mL of tGFP mRNA derived from pTRG2 were incubated at 50, 55, 60 or 65 °C for 90 min (lanes 1-4, respectively).

be the cause of the low expression. Therefore, tGFP gene in which codon usage was optimized for *T. kodakaraensis* was synthesized and mRNA was prepared using a template, pTRG2, which contained the newly synthesized tGFP gene. As a result, about 100-fold increase in tGFP synthesis (as calculated from band intensities of Western blot) was observed (Fig. 2A, lane 2). The author next examined the effect of reaction temperatures on tGFP synthesis within a temperature range from 50 to 65 °C. Western blot analysis of the reaction mixtures showed that the maximum production of tGFP occurred at 60 °C, which corresponded to a tGFP concentration of *ca.* 4.0 μg/mL (Fig. 2B, lane 3).

mRNA stabilization by inserting a stem-loop structure.

Using the *T. kodakaraensis* cell-free translation system, a significant amount of tGFP was synthesized. However, the total amount of tGFP synthesized was still low as compared to the case of ChiAΔ4 (see Chapter 2). When mRNAs used for the synthesis of ChiAΔ4 and tGFP were compared, a difference was found in the length after stop codon: ChiAΔ4 mRNA contained a region corresponding to the *chiA* terminator (363 bases after translational stop codon), while tGFP mRNA had only 7 bases. Hairpin-loop structures are sometimes found in 3' untranslated region of mRNA, serving as a protective barrier to 3' exonuclolytic processing (11). When secondary structure of the *chiA* terminator region was analyzed by the Vienna RNA secondary structure server (http://rna.tbi.univie.ac.at/), several potential stem-loop structures were found, suggesting that the *chiA* terminator region may have a function to stabilize mRNA in the *T. kodakaraensis* cell-free translation system. Therefore, the following two DNA sequences with possible stem-loop structures, the *chiA* terminator region and an artificial sequence pHP10 (2), were selected and inserted at the 3'-end of the tGFP

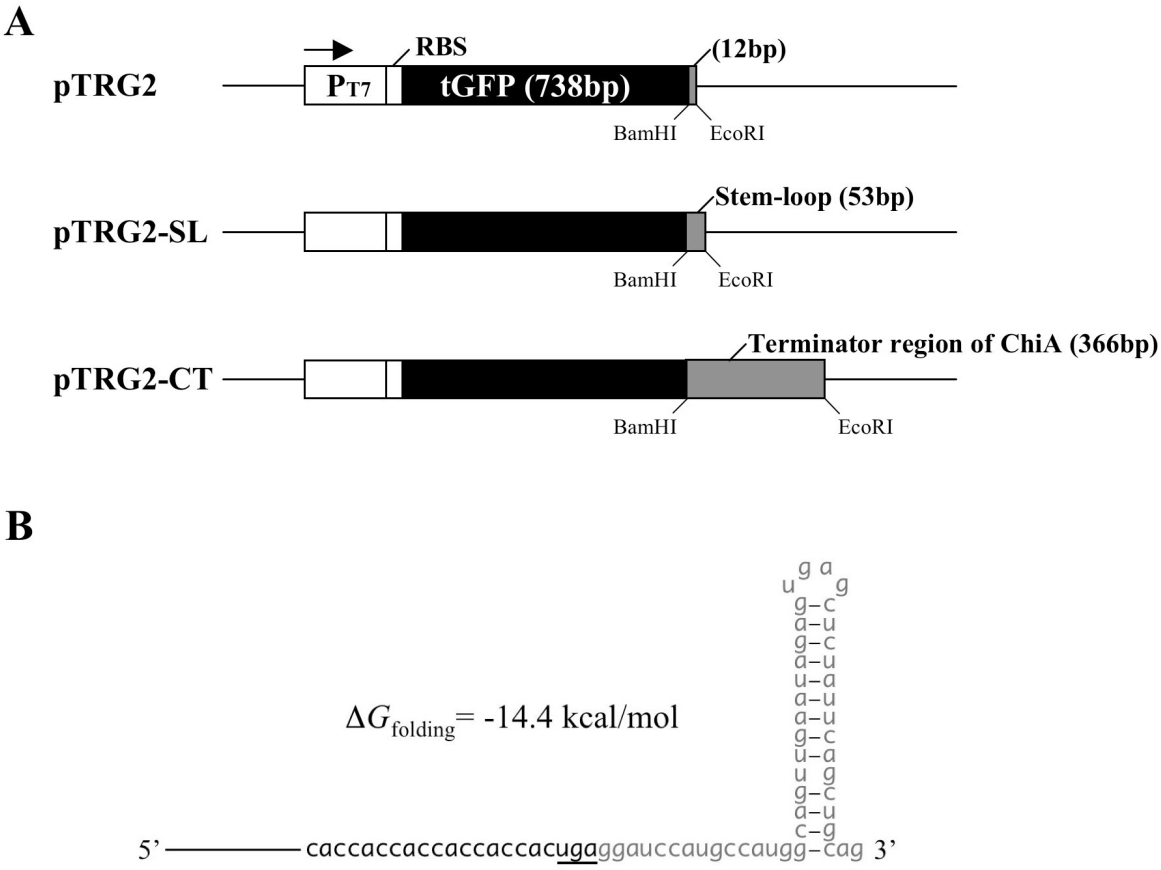
A
pTRG2
RBS
PT7
tGFP (738bp)
(12bp)
BamHI
EcoRI
pTRG2-SL
Stem-loop (53bp)
BamHI
EcoRI
pTRG2-CT
Terminator region of ChiA (366bp)
BamHI
EcoRI
B
ΔGfolding= -14.4 kcal/mol
5'
caccaccaccaccaccacugaggauccaugccaugg-cag
3'
C
1
2
3
4
5
*
tGFP (27 kDa)
D
GFP concentration (μg/mL)
15
10
5
0
pTRG2
pTRG2-SL
pTRG2-CT

Fig. 3 Effect of mRNA 3'-end structure on the tGFP synthesis. (A) Schematic drawings of plasmids, pTRG2, pTRG2-SL and pTRG2-CT, used for preparation of tGFP mRNAs. (B) Predicted 3'-end secondary structure for mRNA derived from pTRG2-SL. Prediction of the secondary structure and calculation of $\Delta G_{folding}$ value (at 60 °C) was done by the Vienna RNA secondary structure server (8). Black and grey characters indicate translated region and untranslated region, respectively, and underline indicates translation stop codon. (C) Detection of tGFP by Western blot analysis. Reaction mixtures containing equimolecular amount of mRNA derived from pTRG2 (0.40 mg/mL, lane 1), pTRG2-SL (0.46 mg/mL, lane 2) or pTRG2-CT (0.57 mg/mL, lane 3) were incubated at 60 °C for 60 min. Reaction mixture with no mRNA was applied as a negative control (lane 4). Purified recombinant tGFP (4.0 μg/mL) added in the reaction mixture for cell-free protein synthesis was applied as a positive control (lane 5). Asterisk indicates a protein band reacted nonspecifically with anti-tGFP antibodies. (D) Total tGFP concentration calculated from band strength of Western blot (open bars) and the active tGFP concentration calculated from fluorescence intensity (filled bars) are indicated as bars in graph. Determinations of tGFP concentrations were from the average of n = 3 reactions and error bars represent standard deviations.

gene to make pTRG2-CT and pTRG2-SL, respectively (Fig. 3A, B). Using equimolecular amount of mRNAs prepared from these templates, cell-free synthesis of tGFP was performed. The production of tGFP increased about 2.5-fold with mRNA containing the *chiA* terminator region (Fig. 3C, lane 3). Insertion of pHP10 sequence was more effective: about 3.5-fold increase in tGFP synthesis was observed (Fig. 3C, lane 2).

Effect of the addition of chaperonins on the production of active tGFP.

The author next examined the ratio of active tGFP present in reaction samples by measuring the fluorescence of tGFP. In all samples tested, the active tGFP constituted *ca*. 30 % of the total tGFP synthesized (Fig. 3D). As tGFP, once folded properly, showed high stability under these conditions (Fig. 1), the results indicated the presence of a heat-labile state(s) in the folding process of tGFP. To increase the efficiency of tGFP folding in the post-translational step, the author next tested the addition of *T. kodakaraensis* chaperonins to the reaction mixtures. Two kinds of chaperonin subunits, CpkA and CpkB, were found in *T. kodakaraensis* (8), and recombinant chaperonin oligomers (16mer) have functions to prevent enzymes from thermal inactivation *in vitro* (7, 16). As chaperonins require ATP for their function, concentration of ATP in the reaction mixture was increased from 4.0 to 5.0 mM. Accordingly, concentration of Mg^{2+} was increased from 3.0 to 4.0 mM, as nucleoside triphosphates (NTP) in solution are present as an NTP-Mg^{2+} complex (1). By the addition of 50 μg/mL of CpkB oligomers, the ratio of active tGFP significantly increased to 48 % while the yield of total tGFP synthesized was comparable (Fig. 4B). By further addition of CpkB oligomers over 100 μg/mL, the amount of tGFP synthesized slightly decreased, while the amount of active tGFP did not change (data not shown). Addition of CpkA oligomers also gave a similar effect: the ratio of active tGFP increased to 42 % in the presence of 50 μg/mL of CpkA oligomers (data not shown).

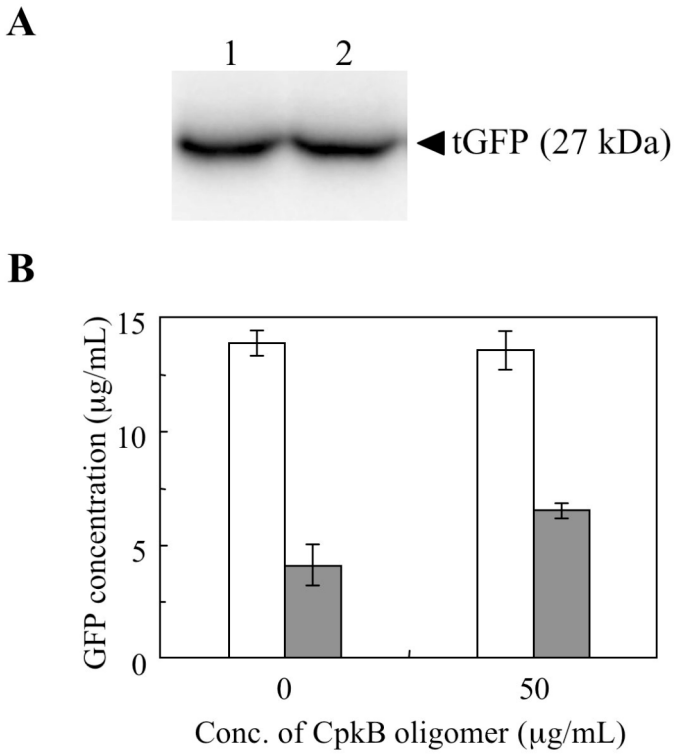

Fig. 4 Effect of the addition of *T. kodakaraensis* CpkB on the tGFP synthesis. (A) Detection by Western blot analysis. Reaction mixtures containing tGFP mRNA derived from pTRG2-CT (0.46 mg/mL) were incubated in the absence (lane1) or presence (lane2) of 50 μg/mL of CpkB oligomers at 60 °C for 60 min, and then separated on the 12.5 % SDS-PAGE gel followed by detection using anti-tGFP antibodies. (B) Total and active tGFP concentrations. Detailed descriptions are as same as those in Fig. 3D.

DISCUSSION

In this chapter, the author succeeded in the synthesis of a heterologous protein, tGFP, using the *T. kodakaraensis* cell-free translation system. The synthesis of tGFP at the initial step was around the levels of detection limit (< 0.1 μg/mL). Using tGFP gene with codons optimized for *T. kodakaraensis*, a large increase in tGFP production was observed, suggesting that codon usage gives a critical effect on the translational

efficiency of the system. At present, as changing codons of an entire gene is relatively easy than before (4), this will be a practical option when proteins of organisms distantly related to *T. kodakaraensis* are produced.

Addition of stem-loop structure(s) at 3'-end of mRNAs also contributed to increase the production of tGFP. In bacterial cells, the presence of stable secondary structure in the 3' untranslated region protects mRNA from degradation by 3' exoribonuclease (11). Accordingly, it was reported that addition of stem-loop structure at the 3'-terminus increased the stability of mRNA in the *E. coli* cell-free translation system (5). Although detailed mechanisms of mRNA decay in *Archaea* are still not known (10), the present result indicated that degradation of mRNA by 3' exoribonuclease also occurred significantly in the *T. kodakaraensis* system. Similar to the case of some bacterial terminators, potential stem-loop structures were found in the *chiA* terminator, and the highest $\Delta G_{folding}$ value among those was -8.81 kcal/mol (at 60 °C). Although addition of the *chiA* terminator was effective to increase the production of tGFP, this $\Delta G_{folding}$ value is higher than that of pHP10 (-14.4 kcal/mol at 60 °C). The difference in the $\Delta G_{folding}$ value may be the reason why pHP10 functioned better than the *chiA* terminator.

The ratio of active tGFP at the initial step was about 30 % of the total tGFP synthesized. As tGFP, once folded properly, has enough stability under the conditions employed, the apparent inconsistency between the yield of protein and the activity clearly suggests the presence of a heat-labile state(s) during folding process of tGFP. With the addition of CpkB oligomers at a concentration of 50 μg/mL, the rate of active tGFP notably increased about 1.6-fold (Fig. 4), while a less significant effect was observed by the addition of CpkA oligomers. It was recently reported in *Thermococcus* sp. KS-1 that oligomers composed of CPNβ (an ortholog of CpkB) have about 14-fold

stronger affinity to prefoldin, which is a molecular chaperone that captures and delivers unfolded proteins to chaperonins, than oligomers composed of CPNα (an ortholog of CpkA) (17). Therefore, the difference in the folding efficiency between CpkA and CpkB oligomers may suggest that nascent polypeptides of tGFP undergo protein folding by a concerted action with prefoldins in the system.

Overall, the present report suggests the possibility of the *T. kodakaraensis* translation system to be used for heterologous protein production. The fact also indicated that this system can be applied as a screening tool to obtain highly thermostable proteins in the future.

REFERENCES

1. **Berg, J. M., J. L. Tymoczko, and L. Stryer.** 2002. Biochemistry 5th ed. W. H. Freeman and Co., New York.

2. **Carrier, T. A., and J. D. Keasling.** 1999. Library of synthetic 5' secondary structures to manipulate mRNA stability in *Escherichia coli*. *Biotechnol. Prog.* **15:**58-64.

3. **Cubitt, A. B., R. Heim, S. R. Adams, A. E. Boyd, L. A. Gross, and R. Y. Tsien.** 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* **20:**448-455.

4. **Gao, W., A. Rzewski, H. Sun, P. D. Robbins, and A. Gambotto.** 2004. UpGene: Application of a web-based DNA codon optimization algorithm. *Biotechnol. Prog.* **20:**443-448.

5. **Hirao, I., S. Yoshizawa, and K. Miura.** 1993. Stabilization of mRNA in an *Escherichia coli* cell-free translation system. *FEBS Lett.* **321:**169-172.

6. **Hoover, D. M., and J. Lubkowski.** 2002. DNAWorks: an automated method

for designing oligonucleotides for PCR-based gene synthesis. *Nucl. Acids Res.* **30:**e43.

7. **Izumi, M., S. Fujiwara, K. Shiraki, M. Takagi, K. Fukui, and T. Imanaka.** 2001. Utilization of immobilized archaeal chaperonin for enzyme stabilization. *J. Biosci. Bioeng.* **91:**316-318.

8. **Izumi, M., S. Fujiwara, M. Takagi, S. Kanaya, and T. Imanaka.** 1999. Isolation and characterization of a second subunit of molecular chaperonin from *Pyrococcus kodakaraensis* KOD1: analysis of an ATPase-deficient mutant enzyme. *Appl. Environ. Microbiol.* **65:**1801-1805.

9. **Kim, T. W., D. M. Kim, and C. Y. Choi.** 2006. Rapid production of milligram quantities of proteins in a batch cell-free protein synthesis system. *J. Biotechnol.* **124:**373-380.

10. **Kulg, G., E. Evguenieva-Hackenberg, A. D. Omer, P. P. Dennis, and A. Marchfelder.** 2007. RNA processing, p. 158-174. *In* R. Cavicchioli (ed.), Archaea: Molecular and Cellular Biology. ASM Press, Washington, DC.

11. **Mott, J. E., J. L. Galloway, and T. Platt.** 1985. Maturation of *Escherichia coli* tryptophan operon mRNA: evidence for 3' exonucleolytic processing after rho-dependent termination. *EMBO J.* **4:**1887-1891.

12. **Nomura, S. M., K. Tsumoto, T. Hamada, K. Akiyoshi, Y. Nakatani, and K. Yoshikawa.** 2003. Gene expression within cell-sized lipid vesicles. *ChemBioChem* **4:**1172-1175.

13. **Sakikawa, C., H. Taguchi, Y. Makino, and M. Yoshida.** 1999. On the maximum size of proteins to stay and fold in the cavity of GroEL underneath GroES. *J. Biol. Chem.* **274:**21251-21256.

14. **Sawasaki, T., T. Ogasawara, R. Morishita, and Y. Endo.** 2002. A cell-free protein synthesis system for high-throughput proteomics. *Proc. Natl. Acad. Sci. USA*

99:14652-14657.

15. **Stemmer, W. P., A. Crameri, K. D. Ha, T. M. Brennan, and H. L. Heyneker.** 1995. Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides. *Gene* **164:**49-53.

16. **Yan, Z., S. Fujiwara, K. Kohda, M. Takagi, and T. Imanaka.** 1997. In vitro stabilization and in vivo solubilization of foreign proteins by the β subunit of a chaperonin from the hyperthermophilic archaeon *Pyrococcus* sp. strain KOD1. *Appl. Environ. Microbiol.* **63:**785-789.

17. **Zako, T., Y. Murase, R. Iizuka, T. Yoshida, T. Kanzaki, N. Ide, M. Maeda, T. Funatsu, and M. Yohda.** 2006. Localization of prefoldin interaction sites in the hyperthermophilic group II chaperonin and correlations between binding rate and protein transfer rate. *J. Mol. Biol.* **364:**110-120.

CHAPTER 4

Synthesis of multiple gene products from a single polycistronic mRNA using the *Thermococcus kodakaraensis*-based translation system

INTRODUCTION

An operon is a genetic unit consisting of a cluster of structural genes and the operator and promoter sequences, and is transcribed as a single unit to produce one polycistronic mRNA. Operons occur primarily in prokaryotes (*Bacteria* and *Archaea*). The first operon described was the *lac*-operon in *Escherichia coli*, by Jacob and Monod in 1961 (3), which is required for the transport and metabolism of lactose. In general, structural genes in the same operon encode proteins sharing a common physiological task. In the case of the *lac* operon, three structural genes (*lacZ*, *lacY*, and *lacA*) encode β-galactosidase, lactose permease, and thiogalactoside acetyltransferase.

Up to now, the author succeeded in the synthesis of a single gene product (ChiAΔ4 and tGFP) using the *T. kodakaraensis* cell-free protein synthesis system. To further develop the *T. kodakaraensis* cell-free protein synthesis system for a broader range of applications, synthesis of multiple gene products is one of the important topics to address. Like the other members in *Archaea*, *T. kodakaraensis* contains operons on its genome (2), suggesting the presence of polycistronic mRNA. In this chapter, the author tested whether multiple gene products from a single mRNA could be synthesized using the *T. kodakaraensis* cell-free system.

MATERIALS AND METHODS

Chemicals.

Sulfur, Tris-acetate, ammonium acetate, polyethyleneglycol 8000 (PEG8000), and potassium phosphoenolpyruvate (PEP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). ATP, GTP, CTP, UTP, 20 amino acids, CoA and hydroxylamine were from Sigma (St. Louis, MO, USA). RNase inhibitor was RNAsecure^{TM} from Ambion (Austin, TX, USA). All the other reagents were obtained from Nacalai Tesque (Kyoto, Japan).

Plasmid construction.

A 220 bp-DNA fragment containing the T7 promoter region and the ribosome-binding site of the *T. kodakaraensis* glutamate dehydrogenase gene (RBS) was amplified from pTRC1 (see Chapter 1) by PCR using the following two primers, T7RBS-N (5'- AAAAGAATTCACTCTAGCTAGAGGATCTCGATCCC -3', underlined sequences correspond to EcoRI site) and T7RBS-C (5'- AAAAGTCGAC TTCCCGTGAGGTTGTAGTACTCAAC -3', underlined sequences correspond to SalI site). After treatment with EcoRI and SalI, the amplified fragment was inserted into the respective sites of pUC118, to make pUC-t7rbs.

A 2,223 bp-DNA fragment containing acetyl-CoA synthetase III operon genes (TK0944 and TK0943) was amplified with genomic DNA of *T. kodakaraensis* KOD1 by PCR using the following two primers, ACS-N (5'- AAAACATATGCACCACCAC CACCACCACTCAGAGAAAATCGTCGAA-3', underlined sequences correspond to NdeI site) and ACS-C (5'- AAAAGTCGACTCAGGCGTAGTCCGGAACGTCGTAC GGGTACTCTTTCTTTTCTGGAGC-3', underlined sequences correspond to SalI site). Nucleotide sequences of these primers were arranged to fuse a hexahistidine-tag

(His-tag) at the amino-terminus of TK0944 protein (His-TK0944) and a hemagglutinin tag (HA-tag) at the carboxyl-terminus of TK0943 protein (TK0943-HA) (Fig. 1B). After treatment with NdeI and SalI, the amplified fragment was inserted into the respective sites of pUC-t7rbs, to make pTACS1.

A 61 bp-DNA fragment containing an artificial stem-loop structure, pHP10 (see Chapter 3) (1), was synthesized using the following two primers, Php-SLN1 (5'-AAAAGTCGACATGCCATGGCAGTTGAATAGAGTGAGCT -3', underlined sequences correspond to SalI site) and Php-SLC1 (5'- AAAAGTCGACTGCAGCT GAATAGAGCTCACTCT -3', underlined sequences correspond to SalI site). The synthesized fragments were treated with SalI, and inserted into the respective sites of pTACS1, to make pTACS1-SL (Fig. 1A).

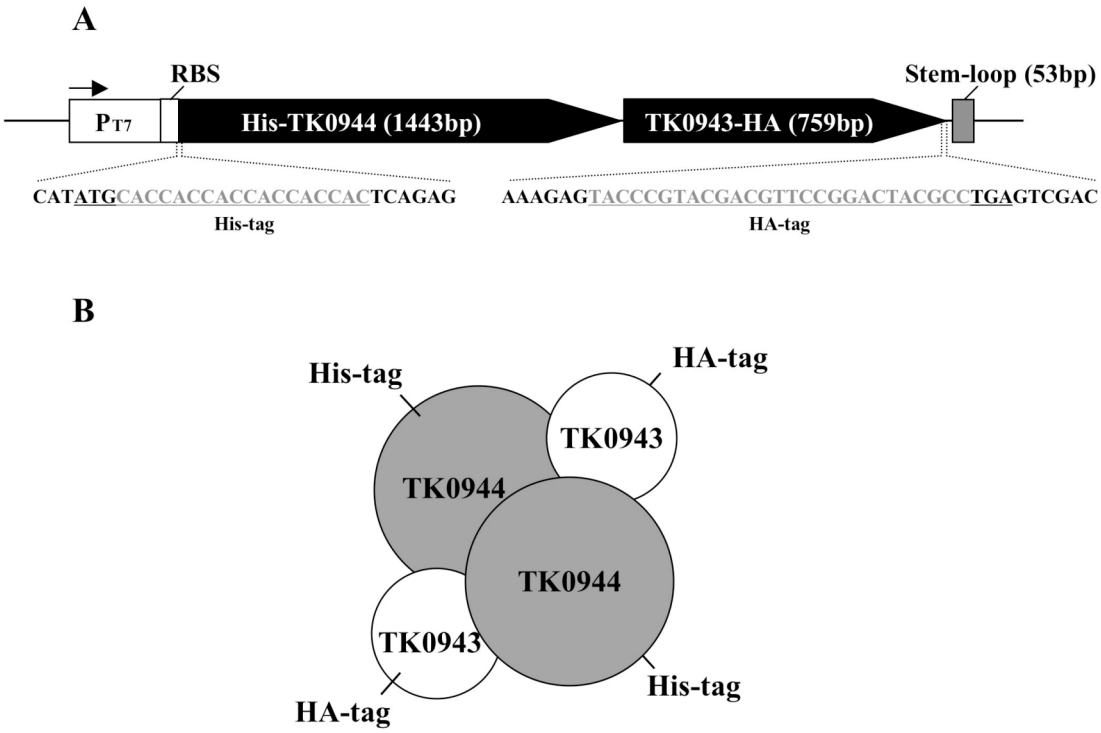

Fig. 1 (A) Schematic drawing of plasmid pTACS1-SL used for preparation of mRNA coding for His-TK0944 and TK0943-HA. (B) Possible structure of a heterotetramer composed of His-TK0944 and TK0943-HA.

mRNA preparation.

A 2,469 bp-DNA fragment containing the T7 promoter region, RBS, and TK0944-TK0943 genes was amplified by PCR using pTACS1-SL as a template and the following two primers, RVS (5'- ACACTTTATGCTTCCGGCTC -3') and Php-SLC1. This fragment was used to prepare mRNA by the T7 RiboMAX^{TM} Express RNA system (Promega, Madison, WI, USA). The synthesized mRNA was suspended in RNase-free water and stored at -80 °C until use.

Reaction conditions for cell-free protein synthesis.

The *T. kodakaraensis* lysate used for cell-free protein synthesis (S30 extract) was prepared as described in Chapter 2 using *T. kodakaraensis* KHR1 strain (*Δphr*). Reactions for protein synthesis were performed at 65 °C for 60 min in a 30 μL mixture containing mRNA (12 μg), *T. kodakaraensis* S30 extract (16 mg/mL), magnesium acetate (4.0 mM), potassium acetate (250 mM), ammonium acetate (80 mM), Tris-acetate (56 mM, pH 8.2), ATP (3.0 mM), GTP (1.5 mM), CTP (1.5 mM), UTP (1.5 mM), PEP (10 mM), PEG8000 (2 %, w/v), spermidine (0.2 mM), 20 amino acids (2.0 mM, each), and RNase inhibitor (RNAsecure^{TM}) (4.0 %, v/v).

Purification of the synthesized protein.

After reactions of protein synthesis, the synthesized protein was purified with the TALONspin^{TM} column (Takara Bio, Kyoto, Japan) for the purification of polyhistidine-tagged proteins. The purified fractions were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5 % acrylamide concentration) and visualized with silver staining.

Western blot analysis.

After cell-free protein synthesis, 0.5 μL of the reaction mixture was analyzed by SDS-PAGE (12.5 % acrylamide concentration). Western blot analysis was performed according to the procedure described in Chapter 1 using rabbit anti-HA antibodies (Sigma).

Acetyl-CoA synthetase assay.

Acetyl-CoA-forming activities were measured by a modified hydroxamate method (4). Reactions were performed in a mixture (500 μL) composed of protein mixture, 2 mM CoA, 20 mM ATP, 100 mM hydroxylamine, 5 mM $MgCl_2$, and 50 mM acetate (pH 6.5) in 140 mM MES-NaOH buffer (pH 6.5). After incubation at 75 °C for 30 min, 350 μL of 20 % trichloroacetic acid and 150 μL of 1 M $FeCl_3$ were added, and formation of the iron-acetylhydroxymate complex derived from acetyl-CoA was determined spectrophotometrically at 520 nm.

RESULTS

Cell-free synthesis of the *T. kodakaraensis* acetyl-CoA synthetase III gene operon.

Acetyl-CoA synthetase catalyzes the formation of acetate from acetyl-CoA and couples this reaction with the synthesis of ATP from ADP and inorganic phosphate. Acetyl-CoA synthetase III of *T. kodakaraensis* (ACSIII) is a heterotetrameric enzyme composed of two α- and two β-subunits ($\alpha_2\beta_2$) (T. Awano, A. Wilming, H. Atomi and T. Imanaka, unpublished data). Genes encoding these subunits constitute a single operon (TK0944-TK0943); where TK0944 encodes the α-subunit and TK0943 encodes the β-subunit.

The ACSIII operon was selected as a target to be synthesized by the *T.*

kodakaraensis cell-free translation system. For the detection and purification of proteins synthesized, template genes were modified to attach a His-tag to the amino-terminus of TK0944 protein (His-TK0944), and a HA-tag to the carboxyl-terminus of TK0943 protein (TK0943-HA) (Fig. 1). With a polycistronic mRNA coding for His-TK0944 and TK0943-HA, cell-free translation was performed at 65 °C for 60 min, and the reaction products were purified using the TALONspin column that selectively attach proteins with His-tag. When the purified fraction was analyzed by SDS-PAGE, a protein corresponding to the molecular weight of His-TK0944 (52.7 kDa) was clearly detected in a mRNA-dependent manner (Fig. 2), suggesting successful synthesis of the first gene

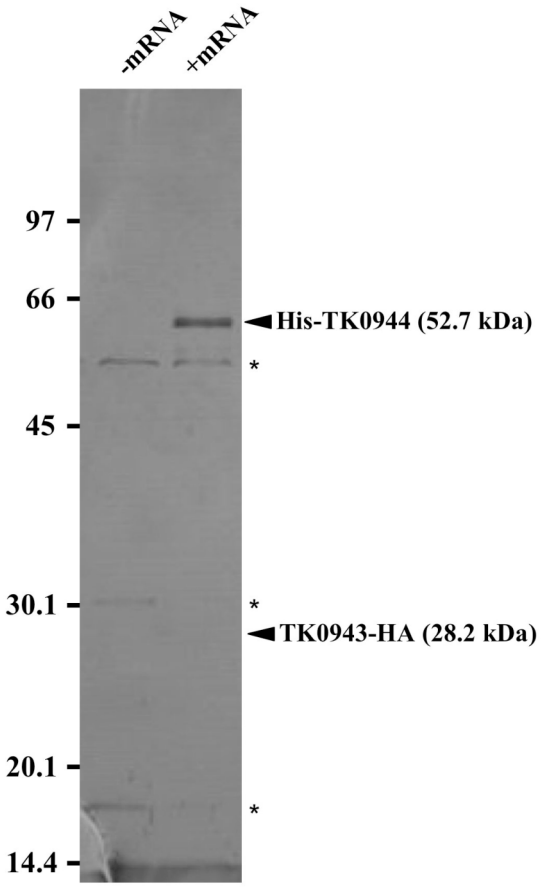

Fig. 2 Purification of His-TK0944 from the reaction mixtures in the presence (+mRNA) or absence (-mRNA) of mRNA coding for His-TK0944 and TK0943-HA. Purified fractions were separated by SDS-PAGE and visualized by the silver staining procedure. Asterisks indicate the protein bands absorbed nonspecifically to the TALONspin columns.

product. As for the second gene product, TK0943-HA protein, if this was synthesized together and formed a stable complex with His-TK0944, this protein should also be present in the purified fraction. However, the protein could not be detected. The TK0943-HA protein simply may not have been synthesized in the cell-free system, or the result may have been due to the fact that the interaction between the two proteins was not strong enough for the two to maintain contact during the purification procedures.

To determine whether or not the TK0943-HA protein was synthesized, Western blot analysis was performed for the reaction mixtures using anti-HA-tag antibodies. As a result, a band corresponding to the molecular weight of TK0943-HA (28.2 kDa) was clearly detected (Fig. 3), indicating successful synthesis of both gene products (His-TK0944 and TK0943-HA) from a polycistronic mRNA by the *T. kodakaraensis* cell-free translation system.

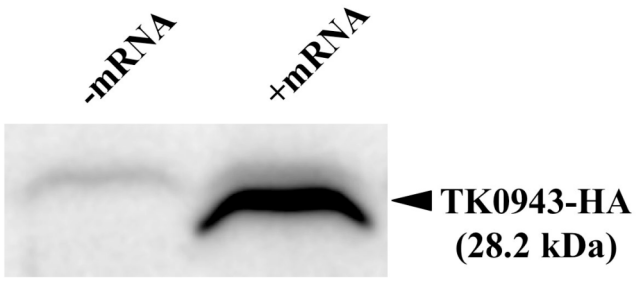

Fig. 3 Detection of TK0943-HA by Western blot analysis using the reaction mixtures in the presence (+mRNA) or absence (-mRNA) of mRNA. The TK0943-HA synthesized was detected using polyclonal anti-HA-tag antibodies.

Table 1. ACS activities in the reaction mixtures

	-RNA	+RNA
Absorbance (520 nm)	**0.568±0.014**	**0.564±0.017**
	(100 %)	**(99.3 %)**

Results are the average of n = 5 reactions.

As the synthesis of both ACS subunits were confirmed, ACS activities in the reaction mixtures were determined. ACS activities in the mixtures with or without the presence of mRNA were examined for ATP-dependent acetyl-CoA formation by the hydroxamate method. However, even in the mixture without mRNA, considerable ACS activities were detected, and no significant difference in activities could be detected by the presence or absence of mRNA (Table 1).

DISCUSSION

In this chapter, the author attempted to synthesize two proteins (His-TK0944 and TK0943-HA) that potentially form a heterooligomeric complex, from a single polycistronic mRNA using the *T. kodakaraensis* cell-free translation system. Successful synthesis of both proteins was confirmed, but the complex formation of these proteins was not detected. There may be several reasons for this. The first one is that tag regions fused to these proteins might interfere with complex formation. The crystal structure of acetyl-CoA synthetase II from *Pyrococcus horikoshii* has been determined for dimeric α-subunits (PH0766: Protein Data Bank code 2CSU) and a monomeric β-subunit (PH1788: Protein Data Bank code 1WR2), independently. From these structures, Shikata *et al*. illustrated how these subunits are arranged (4). According to their prediction, a His-tag attached to the amino-terminus of TK0944 would be located

outside of the complex, while the location of a HA-tag attached to the carboxyl-terminus of TK0943 is not clear and may inhibit complex formation. Therefore, the author also tested the synthesis of His-TK0944 protein with the wild-type TK0943 protein. However, in this case also, no complex formation was observed when His-TK0944 proteins were purified using the His-tag (data not shown), showing that the fusion of the HA-tag is not the major reason for the inability to form a complex.

The second reason may be the limited affinity between TK0944 protein and TK0943 protein. Recently, assembly of the β-subunit protein (TK0943) with multiple α-subunits (TK0944, TK1880, TK0139, and TK2127) was suggested *in vivo* (4). As the reaction mixture contains multiple α-subunits in a large amount originating from the cell-free (S30) extract, it can be presumed that the synthesized TK0943-HA protein does not bind exclusively to the synthesized His-TK0944 protein.

REFERENCES

1. **Carrier, T. A., and J. D. Keasling.** 1999. Library of synthetic 5' secondary structures to manipulate mRNA stability in *Escherichia coli*. *Biotechnol. Prog.* **15:**58-64.

2. **Fukui, T., H. Atomi, T. Kanai, R. Matsumi, S. Fujiwara, and T. Imanaka.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. *Genome Res.* **15:**352-363.

3. **Jacob, F., and J. Monod.** 1961. Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* **3:**318-356.

4. **Shikata, K., T. Fukui, H. Atomi, and T. Imanaka.** 2007. A novel ADP-forming succinyl-CoA synthetase in *Thermococcus kodakaraensis* structurally

related to the archaeal nucleoside diphosphate-forming acetyl-CoA synthetases. *J. Biol. Chem.* **282:**26963-26970.

GENERAL CONCLUSIONS

In this thesis, the author developed a novel cell-free protein synthesis (CFPS) system based on the hyperthermophilic archaeon, *Thermococcus kodakaraensis*. The author further applied this system in the field of heterologous protein production. The overall summary of this thesis is shown in Fig. 1 and Table 1.

Development of the T. kodakaraensis CFPS system

In Chapter 1, the author described the development of the *T. kodakaraensis* CFPS system that can be operated in a temperature range between 40 °C and 80 °C (optimum temperature is 65 °C). Using the system, a ChiAΔ4 protein, a derivative of *T. kodakaraensis* chitinase, was produced in an active form. The author also succeeded in a CFPS reaction coupled with the T7 RNA polymerase-dependent transcription reaction. In Chapter 2, the author further developed the system to increase its productivity through 1) improvements in cell lysate preparation, 2) optimization of reaction mixture compositions, and 3) genetic alteration of the host strain. As a result, the yield of ChiAΔ4 reached 115.4 μg/mL in a batch reaction at 65 °C. Moreover, in the optimized system, a high rate of protein synthesis was achieved: over 100 μg/mL of ChiAΔ4 was produced in the first 15 min of the reaction, which is comparable to the levels of the *E. coli* system. On the other hand, a relatively short duration of protein synthesis (~ 30 min) was also observed, and resolving this problem will be necessary to further increase the protein yield.

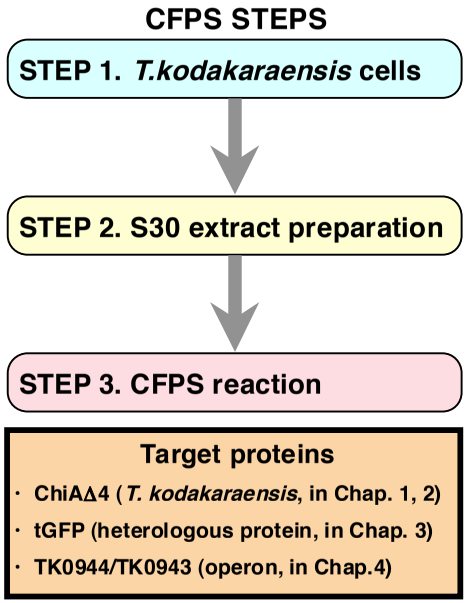

Fig. 1 Flow chart of the CFPS steps.

Table 1. Approaches taken to improve the *T. kodakaraensis* CFPS system.

Step	Approaches	Effects	Chapter
1	Genetic alteration (Δ*phr* strain)	Reaction temperature ↑ Protein yield ↑	2
2	Optimization of FP* conditions	Activity of S30 extract ↑	2
	Omission of the pre-incubation step	Activity of S30 extract ↑	2
3	Optimization of mixture compositions	Translation activity ↑	1, 2
	Codon optimization	Translation efficiency ↑	2
	Addition of stem-loop structure	mRNA stability ↑	3
	Addition of chaperonin	Protein folding efficiency ↑	3

*FP: French press

Application of the T. kodakaraensis cell-free protein synthesis system

The author next applied the *T. kodakaraensis* CFPS system in the field of protein production. In Chapter 3, a thermostable green fluorescent protein (tGFP) was produced in a fluorescence-active form. In Chapter 4, multiple gene products (TK0944/TK0943) were synthesized from a single polycistronic mRNA.

Three effective points for the synthesis of active tGFP with the *T. kodakaraensis* cell-free system were identified, which are as follows: 1) the addition of a stem-loop structure at the 3'-end of mRNA, 2) the optimization of codon usage, and 3) the addition of chaperonin oligomers. The positive effects brought about by these modifications provide valuable information for future synthesis of heterologous proteins using the system. Moreover, successful synthesis of heterologous proteins using the *T. kodakaraensis* CFPS system indicated that this system could be applied as a screening tool to obtain highly thermostable proteins in the future.

LIST OF PUBLICATIONS

1 Cell-free protein synthesis at high temperatures using the lysate of a hyperthermophile.

Takashi Endoh, Tamotsu Kanai, Yuko T. Sato, David V. Liu, Kenichi Yoshikawa, Haruyuki Atomi, and Tadayuki Imanaka.

Journal of Biotechnology. 2006. **126** (2), 186-195.

2 A highly productive system for cell-free protein synthesis using a lysate of the hyperthermophilic archaeon, *Thermococcus kodakaraensis*.

Takashi Endoh, Tamotsu Kanai, and Tadayuki Imanaka.

Applied Microbiology and Biotechnology. 2007. **74** (5), 1153-1161.

3 Effective approaches for the production of heterologous proteins using the *Thermococcus kodakaraensis*-based translation system.

Takashi Endoh, Tamotsu Kanai, and Tadayuki Imanaka.

Journal of Biotechnology. 2008. **133** (2), 177-182.

4 Synthesis of multiple gene products from a single polycistronic mRNA using the *Thermococcus kodakaraensis*-based translation system.

Takashi Endoh, Tamotsu Kanai, and Tadayuki Imanaka.

In preparation.